Isolation and Analysis of a *Coprinus cinereus* DNA Fragment Showing Homology to a Fimbrial cDNA from *Ustilago violacea*.

by

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Abstract

Surface proteinaceous fibrils, termed fimbriae, were first identified on gram negative bacteria in the 1940s. Fungal fimbriae, discovered some 25 years later, are found on members of all fungal classes. In the present study, polyclonal antiserum raised against the fimbrial proteins of *U. violacea* were used in order to identify antigenically related proteins from *Coprinus cinereus* and *Schizophyllum commune*. Two polypeptides with molecular masses of 37 and 39 kDa from *C. cinereus* were observed and confirm earlier results. A single previously unidentified 50 kDa polypeptide in *S. commune* crossreacted with the antiserum. The 50 kDa protein was found to consist of 3 isoforms with isoelectric points ranging from 5.6 to 5.8.

A fimbrial cDNA derived from *U. violacea* was used to identify DNA restriction fragments from *C. cinereus* and *S. commune* showing homology to the fimbrial transcript of *U. violacea*. Heterologous hybridization with this cDNA was used in order to screen a *C. cinereus* genomic DNA library. A single clone, λ2-3A, with a 14 kbp insert showed strong homology to the pfim3-1 cDNA. The region of homology, a 700 bp *Xba* I fragment, was subcloned into pUC19. This plasmid was refered to as pXX8. DNA sequence determinations of pXX8 and adjacent fragments from λ2-3A suggested that the cloned DNA was a portion of the rDNA repeat encoding the small subunit rRNA.

DNA sequence analysis of pfim3-1 yielded an incomplete open reading frame. The predicted amino acid sequence codes for a 206 amino acid, 22 kDa polypeptide which contains a domain similar to a transmembrane domain from rat leukocyte antigen, CD53. As well, an

untranslated 576 nucleotide domain showed 81% homology to pXX8 and 83% homology to the 18S rRNA sequence of *Ustilago maydis*. This sequence was found adjacent to a region of adenine-thymine base pairs presumed to represent the polyadenylation sequence of the fimbrial transcript. The size and extent of homology is sufficient to account for the hybridization of pfim3-1 to rDNA. It is suggested that this domain represents a completely novel regulatory domain within eukaryotes that may enable the observed rapid regeneration of fimbriae in *U. violacea*.

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Table of Contents

List of Illustrations

List of Commonly Used Abbreviations

AU: antiserum raised against fimbrial proteins of *Ustilago violacea*.
bp: base pairs.
cDNA: complementary DNA.
Da: dalton
FASTA: sequence analysis program.
IEF: isoelectric focusing.
gd H_2O: glass distilled water (sterilized).
kbp: 1000 base pairs.
mRNA: messenger RNA
N-AGA: N-acetylglucosamine
N-MePhe: N-terminal methylated phenylalanine.
NS: preimmune serum.
nt: nucleotide.
OD: optical density.
ORF: open reading frame.
pfu: plaque forming unit.
pI: isoelectric point.
poly-A: poly-adenylation sequence.
rDNA: DNA encoding ribosomal RNA.
r-protein: ribosomal protein.
rRNA: ribosomal RNA.
S: Svedbergs (sedimentation coefficient)
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.
TSP: total soluble proteins.
3': end of nucleic acid with OH at carbon 3 of terminal nucleotide.
5': end of nucleic acid with PO_4 at carbon 5 of terminal nucleotide.

Introduction

The role of fimbriae has been investigated in a number of fungi. These roles include flocculation in *Saccharomyces cerevisiae* (Day et al., 1975), conjugation in *Ustilago violacea* (Day and Poon, 1975; Day, 1976) and virulence in *Piptocephalis virginiana* (Rghei et al., 1992). The reported functions of fimbriae in these fungi are conceptually similar to contact events characteristic of filamentous fungi, specifically basidiomycetes. These include a number of hyphal fusion events: self anastomosis, sexual anastamosis and clamp connection formation. Evidence implicating fimbriae in contact events is only circumstantial as attempts to generate afimbriate *U. violacea* mutants has yielded strains which revert to wild type (Gardiner, 1985). The mechanism of reversion is unknown.

An alternative approach to the generation of afimbriate mutants involves the cloning of fimbrial genes. Cloned genes could be used to alter expression of fimbrial genes in transformed cells and to examine the regulation of fimbrial gene transcription. Fimbrial cDNAs derived from *U. violacea* (Castle, personal communication) could provide a means for the isolation of homologous fimbrial genes from other fungi.

Coprinus cinereus and *Schizophyllum commune* are Basidiomycetes with well characterized transformation systems (Binninger et al., 1987; Munoz-Rivas et al., 1986; Specht et al., 1988). The existance of fimbriae has been confirmed on both *C. cinereus* and *S. commune* (Boulianne, 1990). Additionally, the fimbrial subunit of *C. cinereus* has been identified using a polyclonal antiserum raised against the fimbrial proteins of *U. violacea*. Preliminary results suggest that fimbrial cDNAs from *U. violacea* hybridize to genomic DNA from a variety of

fungi, including *C. cinereus* and *S. commune* (Castle, personal communication). For the above mentioned reasons, *C. cinereus* and *S. commune* were selected as study organisms for the cloning and characterization of fimbrial genes in view of ultimately initiating further studies on fimbrial gene expression and function.

The objectives of the current research were to:

1. Identify and partially characterize proteins of *C. cinereus* and *S. commune* that share epitopes with the fimbrial proteins of *U. violacea*.
2. Test whether or not fimbrial cDNAs derived from *U. violacea* hybridize with genomic DNA from *C. cinereus* and *S. commune*.
3. Clone and determine the nucleic acid sequence of a segment of DNA from either *C. cinereus* or *S. commune* showing homology to fimbrial cDNAs.

Literature Review

Bacterial Fimbriae and Pili

Non flagellar surface fibrils were first observed on bacteria following the development of electron-microscopic techniques (Anderson, 1949; Houwink and van Iterson, 1950). These structures have been referred to as threads, bristles, filaments, fibrillae, colonization factor antigens, adhesins, fimbriae and pili (Reviewed in Paranchych and Frost, 1988). The term "fimbria" (Duguid et al, 1955), Latin for thread or fibre, and the term "pilus" (Brinton, 1959), Latin for hair, are currently the terms often used interchangeably to designate these extracellular proteinaceous appendages. However, some authors propose a distinction between fimbriae and pili based on functionality (Reviewed in Ottow, 1975). Thus, "pili" refer to structures encoded by plasmids involved in bacterial conjugation whereas the term "fimbriae" is reserved for those non conjugative appendages, either chromosomally coded or plasmid encoded. Pili and fimbriae will be thus distinguished in this review.

Fimbriae were first discovered on members of the Enterobacteriacea. They were subsequently found to occur elsewhere among the gram-negative bacteria and to a lesser extent in the gram-positive bacteria. Pili and fimbriae have been studied predominantly within the Enterobacteriacea and thus this bias shall be reflected in this review.

Structure

Bacterial fimbriae and pili have been found to vary both among strains and within an strain. Several classification schemes have been

proposed with as many as seven categories of fibrils (Duguid, 1968). Currently, there is no scheme which is universally excepted. In the present review, five broad groups will be examined here which are distinguished based on morphology, binding specificity and function. The five fimbrial types represented here are well documented in the literature due to the economic importance of the bacteria from which these have been isolated. In no way is it suggested that this scheme encompasses all fimbrial types.

The first group is composed simply of the "common" or the "type 1 pili" of Duguid (1968). These are adhesive fimbriae expressed on an estimated 70% of all wild type *Escherichia coli* isolates (Reviewed in Klemm, 1985). *E. coli* with the type 1 phenotype bind guinea pig erythrocytes in a D-mannose sensitive manner (mannose sensitive hemagglutination). The specific receptor bound by these fimbriae are mannose residues of glycoconjugate macromolecules at the cell surface (Reviewed in Klemm, 1985). These fimbriae are rigid rodlike structures approximately 7 nm in diameter with an axial hole of 2 nm in diameter. The 157 amino acid containing subunits are arranged in a helical manner with 3.125 subunits per revolution and a pitch distance of 2.3 nm (Reviewed in Klemm, 1985).

A second group of fimbriae are morphologically similar to type 1 but do not show the mannose sensitive hemagglutination. This group includes fimbriae from both enterotoxigenic strains and uropathogenic strains. The former include the colonizing factor antigens CFA1, CS1 and CS2 (Reviewed in Klemm, 1985). These have been implicated in the binding of *E. coli* to human intestinal epithelium. Among the fimbriae isolated from uropathogenic strains are P (bind P blood group antigens),

Pap (pyelonephritis-associated pili) and F1C (Reigman *et al.*, 1990). Mannose resistant hemagglutinating fimbriae bind α-D-galactopyranose(1-4)ß-D-galactopyranoside (Galα1-4Gal) (Reviewed in Klemm, 1985). All P-related fimbriae including those from *E. coli* strains isolated from dog bind Galα1-4Gal but show variation in the specificity of binding to cell receptors. Variability has been attributed to the differential binding to residues adjacent to the galactoside dimer in cell surface glycoconjugates (Stromberg et al., 1990).

A third group of fimbriae are made up of slender nonhelical fibrils, approximately 2nm in diameter (Reviewed in Klemm, 1985). Fimbriae in this group include K88, K99, F41 and CS3. These were all isolated from enterotoxigenic *E. coli*. The subunit sizes vary within this group: 27.5 kilodaltons (kDa), 29.5 kDa and 18.4 kDa for K88, F41 and K99, respectively. Fimbrial subunits in general are acidic, however, the K99 fimbrial subunits have a pI of 9.5 making it the only basic fimbrial subunit protein observed to date (Reviewed in Klemm, 1985).

The K88 fimbriae have been subdivided into K88ab, K88ac and K88ad based on their antigenicity. The a antigen is conserved whereas the b, c and d antigens are variable. These differences seem to be reflected in the amino acid sequence between residue 80 and the C terminus (Reviewed in Klemm, 1895). The conserved N-terminus is reported to be involved in subunit polymerization (Reviewed in Mooi and DeGraaf, 1985).

The fourth group are the fimbriae with a conserved methylated phenylalanine residue at the N terminus (N-MePhe). These are not found in the enterobacteriaceae (Elleman, 1988). They are prominent on *Neisseria gonorrhoeae*, *N. meningitidis*, *Actinobacter calcoaceticus*,

Eikenella corrodens, *Moraxella bovis*, *M. nonliquefaciens* and *Bacteroides nodosus*. These fimbriae are 5-6nm in diameter and up to 10μm in length (Elleman, 1988). They are helical with 5.1 subunits per revolution and a pitch of 41nm. The subunits are between 16 and 18 kDa depending on the isolate and are α-helical with the axis parallel to the axis of the fibre (Elleman, 1988).

In *B. nodosus* 8 serogroups have been determined, A-H, which fall into two sets, the A set (A,B,C,E,F and G) and the D set (D and H) (Elleman, 1988). Serotypes in the A set show between 60% and 80% homology whereas those in the D set show 68% homology, based on deduced amino acid sequences. A and D sets show less than 40% homology (Elleman, 1988). The conserved sequences fall almost exclusively at the N-terminus containing the N-MePhe residue. The first 32 amino acids are highly conserved and have been proposed as the subunit-subunit binding site (Elleman, 1988). In *N. gonorrhoeae* and *N. meningitidis,* the residues 33-55 are also somewhat conserved and have been implicated in receptor binding (Elleman, 1988).

The fifth group is comprised of the conjugative or sex pili. These are encoded in the transfer region of self transmissable plasmids. Following release of pheromones by the donor cell, mating aggregates are formed. Sex pili recognize the recipient cell and mediate attachment, replication and transfer of the plasmid (Paranchych and Frost, 1988). The inability of closely related sex pili, encoding plasmids, to co-reside in a host has been used to classify pili types into incompatibility groups (20 in *E. coli*). These show similarity within the groups in terms of plasmid characteristics, pilus characteristics and genetic markers such as resistance to antibiotics

and heavy metals (Paranchych and Frost, 1988). F-pili, one incompatibility group, have been found to be 8nm in diameter with a 2nm axial hole. The fibre is composed of subunits forming a helix with 12.5 units per revolution and a pitch distance of 16nm (Marvin and Folkhard, 1986).

Function

Various functions have been attributed to bacterial fimbriae and pili. The earliest role attributed to these fibrils was adhesion. It has long been realized that fimbriae agglutinate erythrocytes (Duguid, 1955). Hemagglutination characteristics have been used as a means of classification of fimbriae. The various fimbrial types, as previously mentioned, bind specific carbohydrate residues of glycosylated macromolecules (Reviewed in Klemm, 1985).

The binding of fimbriae to cell surfaces has been correlated with the virulence of a number of pathogens. *Pseudomonas aeroginosa* adhesion to respiratory epithelial cells has been inhibited by pretreatment of bacteria with monoclonal antibodies which recognize the fimbriae of *P. aeroginosa* (Doig et al, 1990). The uptake of nutrients and the release of heat labile enterotoxins has been correlated with the presence of fimbriae making fimbriate strains 100 times more virulent than afimbriate strains (Eisenstein, 1988). Fimbriae are thought to bind epithelial cells allowing establishment of infection.

Infant senepsis and meningitis can be initiated through oropharyngeal infection of *E. coli*. The development of type 1 fimbrial mutants has allowed the determination of the role of fimbriae in

establishment of these infections. Fim$^-$ mutants, lacking fimbriae, were unable to colonize the throat (Bloch and Orndorff, 1990). It appears that the specific adherence mediated by fimbriae is a determinant in colonization and thus of virulence.

It has also been determined that fimbriae can be important in the nonspecific adherence to solid substrates. N-MePhe fimbriae of *P. aeruginosa* are involved in adherence to both stainless steel and polystyrene as determined through adherence inhibition studies using monoclonal antibodies directed against fimbriae (Irvin, 1990). This does not exclude N-MePhe fimbriae from playing a role in virulence. Ovine footrot develops only after successful colonization by fimbriated strains of *Bacteriodes nodosus* (Elleman, 1988) suggesting a role in pathogenesis.

The N-MePhe fimbriae of *N. gonorrhoeae* mediate competence of cells to the transformation of DNA (Seifert and So, 1988). Fim$^-$ cells exhibit low transformation efficiencies (Seifert et al., 1990). This inefficiency can be overcome in part by addition of $CaCl_2$ which presumably overcomes the repulsion of like charges on the cell surface and the DNA. Presence of fimbriae reduces these charge effects increasing uptake of foreign DNA (Seifert et al., 1990). Transformation of *N. gonorrhoeae* has been shown to be a mechanism for increasing antigenic variation in fimbriae (Seifert and So, 1988). Antigenic variation in itself may be a mechanism for the avoidance of a host immune response.

N-MePhe fimbriae are capable of antigenic variation through a variety of mechanisms. *Neisseria gonorrhoeae* fimbrial genes contain variable regions which are capable of undergoing recombination with

silent loci to yield antigenic variants that evade the human immune response (Hagblom et al, 1985). *Moraxella bovis,* which gives rise to bovine keratoconjunctivitis, has been shown to vary fimbrial phenotype through an inversion mechanism (Marrs et al., 1988). These mechanisms will be discussed further at a later stage.

Conjugative or sex pili mediate the attachment between donor and recipient cells in conjugation (Achtman et al, 1978; Brinton et al., 1964). Conjugation will not occur if pilus assembly is halted or if pili are mechanically removed or damaged (Willetts and Skurray, 1980; Novotny et al., 1969). The transfer of the F pilin coding plasmid occurs following pilus attachment to the recipient. Two hypotheses exist for the transfer of DNA. Brinton (1965) suggested that the transfer may be made through the axial hole in the pilus without the necessity of close contact. Curtiss (1969) suggested that the pilus may be dissassembled in such a way that the donor and recipient come into contact and establish a cytoplasmic continuity. These two proposed mechanisms may not be mutually exclusive as the pilus may constitute part of a cytoplasmic bridge (Ippen-Ihler and Minkey, 1986).

Accessory Proteins

A number of proteins appear to be required for the expression and proper assembly of fimbriae. These accessory proteins have been described for fimbriae with diverse antigenicity including the *E. coli* type 1, K88ab and Pap fimbriae.

Seven proteins involved in the synthesis of type 1 fimbriae have been examined: Hyp (23 kDa), Pil A (17 kDa), Pil B (30 kDa), Pil C (86 kDa), Pil D (14 kDa), Pil E (31 kDa) and Pil F (18 kDa) (Maurer and

Orndorff, 1985; Maurer and Orndorff, 1987). The function of a number of these have been elucidated. The Pil A protein is the subunit protein mentioned previously. Pil A^- phenotype lacks fimbriae entirely but hemagglutination may occur (Maurer and Orndorff, 1985). The Pil E protein has been shown to be an adhesin that gives the receptor binding specificity to the fimbrial structure. A Pil E^- phenotype shows morphologically indistinguishable fimbriae from type 1, however, these will not show hemagglutination (Maurer and Orndorff, 1985). These two proteins make up the fimbrial fibre.

The length of fimbriae seems to be regulated by the Pil F protein. Pil F^- *E. coli* show a three fold increase in fimbrial length, on average, as well as a slight decrease in the number of pili per cell. The relative proportion of Pil F (1/100 Pil A typically) dictates the length of fimbriae as this highly homologous protein seems to be a competitive inhibitor of Pil A subunit binding (Maurer and Orndorff, 1987). Pil F may also be involved in the anchoring of fimbriae to the cell surface. The roles of the remainder of the accessory proteins for type 1 fimbriae are not yet entirely clear.

Fimbriae obtained from enterotoxigenic strains of *E. coli* similarly have numerous proteins involved in fimbrial assembly. These are well characterized for the K88ab fimbriae. These have been named based on their molecular weight in kilodaltons (kDa): p26, p17.6, p27, p17, p27.5 and p81 (Reviewed in Mooi and DeGraaf, 1985). The p26 protein is the major structural subunit of this fimbrial type. It appears that p17.6 is a minor structural component. The roles of the other four proteins have been attributed to subunit transport and assembly as determined by the construction of mutants for each of these proteins through transposon

mediated mutagenesis of the corresponding genes (Reviewed in Mooi and DeGraaf, 1985).

The p81 deficient mutants were found to be without fimbriae. There was an accumulation of p26 structural subunits in the periplasm loosely associated with p27 (Reviewed in Mooi and DeGraaf, 1985). It is thought that this association with p27 is a transient complex (p26-p27) to shuttle subunit proteins through the periplasm in such a way that the subunits do not spontaneously form fibrils. The pIs of p26 and p27 are acidic (pI=4.2) and basic (pI=9.3), respectively, offering support for an ionic interaction between these proteins (Reviewed in Mooi and DeGraaf, 1985). Additionally, p17 was also found to be associated with p26-p27 in the periplasmic space in $p81^-$ strains.

Generation of $p17^-$ mutants led to expression of fimbriae which lacked the mannose resistant hemagglutination phenotype. There also tended to be more p26 in the periplasm than that found in wild type strains (Reviewed in Mooi and DeGraaf, 1985). It has been proposed that the p17 protein is involved in both transport through the periplasmic area and conformational changes in the subunit required for adhesive properties of fimbriae. An alternative view is that p17.6 is an adhesin occurring in the fimbriae and that this may be shuttled through the periplasm complexed to p17 (Reviewed in Mooi and DeGraaf, 1985).

Nontransport of the fimbrial subunits to the outer membrane among strains expressing the $p81^-$ phenotype and localization of the p81 protein at the cell surface, as determined with antisera raised against this protein, offer support for the p81 protein acting as a specific channel for the transport of fimbrial subunits across the outer

membrane (Reviewed in Mooi and DeGraaf, 1985). This transport may also mediate conformational changes in p26 such that the spontaneous assembly of the fimbriae may occur.

The role of p27.5 is as yet undetermined. It is localized within the periplasmic space and thus may play a role in stability, transport or conformational changes in fimbrial proteins during export (Reviewed in Mooi and DeGraaf, 1985).

Fimbriae isolated from uropathogenic strains of *E. coli* express numerous Pap proteins. Nine Pap proteins have been identified (Pap A-Pap I) (Parachych and Frost, 1988). Pap A is the structural protein fimbrillin (16.5 kDa). Expression of this protein is regulated at the level of transcription by the Pap I and Pap B proteins. Pap C (81 kDa) and PapD (28.5 kDa) have been implicated in the export and stability of Pap A in a similar way to the K88ab system described.

Differences arise in the Pap E, Pap F and Pap G proteins. These form a terminal complex on Pap fimbriae which confers the specificity of binding (Lindberg *et al.*, 1987). Pap G is the specific adhesin whereas Pap E and Pap F are involved in the attachment of Pap G to tip of the fimbriae. Variation in the primary sequence of G adhesins from different Pap fimbriae producing strains appears to affect the specificity of binding. Antigenically different Pap fimbriae bind α-D-galactopyranoside(1,4)ß-D-galactopyranoside with varying specificity. The residues adjacent to the digalactoside on the recognized glycoconjugate are differentially recognized by amino acids encoded in variable domains within *pap G* (Stromberg et al, 1990).

Pap H protein was found to be highly homologous to Pap A with the exception of the addition of a proline rich 14 amino acid sequence at

the N-terminus. Similar extensions of staphylococcal protein A and streptococcal M protein have been implicated in cell wall binding (Baga et al, 1987). Pap H^- strains appear to have 50%-70% of their fimbrial subunits detached from the cell surface (Baga et al, 1987). Similar to the Pil F protein in type 1 fimbriae, Pap H appears to be both a competitive inhibitor of fimbrial polymerization and an anchoring protein. The binding of Pap H to Pap A inhibits further addition of subunits at the base yielding a mechanism for length modulation based on the relative abundance of these proteins. The proline rich extension appears to be associated with the cell surface and thus would act as an anchoring structure for fimbriae (Baga et al, 1987).

Regulation

Most of the bacteria investigated with respect to fimbriae and pili are mammalian pathogens which must both be virulent at physiological temperatures and evade the host immune response in order to be successful. Fimbriae, which are exposed structures, must necessarily be regulated for the bacterium to have a selective advantage. A number of fimbrial regulatory mechanisms will be examined with respect to the physiological roles they play.

Pathogens of mammals must be capable of infection at physiological temperature. It would be favourable for virulence determinants to be expressed under the conditions of infection. Enterotoxigenic *E. coli* strains show temperature sensitive regulation of K88 fimbriae (Reviewed in Mooi and DeGraaf, 1985). DNA sequence information of the fimbrial operon shows an area of dyad symmetry at the 5' end of the gene encoding p81 protein. The initiation site for translation of the

p81 transcript is found within this sequence. At low temperature, the symmetry of these sequences result in the formation of a hairpin loop which inhibits translation (Reviewed in Mooi and DeGraaf, 1985). Genes encoding other fimbrial proteins are distal to the p81 gene thus the interruption of translation is also expected to affect expression of these downstream structural proteins.

Phenotypic changes between fimbriate (Fim^+) and nonfimbriate (Fim^-) strains within a single *E. coli* colony have been reported at a frequency of about 1 switch per thousand generations for type 1 fimbriae (Eisenstein, 1988). This rate is far greater than the spontaneous mutation rate and the change has been termed phase variation. DNA sequence information from Fim^- strains was compared to Fim^+ expressing strains. A 314 base pair (bp) invertable sequence with 9 bp terminal inverted repeats mediates the phase variation (Abraham et al, 1985). This sequence contains the promoter for the fimbrial operon and thus regulates transcription.

This system is acted upon by five trans-acting genes. *pil* B and *pil* E are located near *pil* A (the structural protein) (Spears et al., 1986). The presence of a distant *pil* G gene (Reviewed in Klemm, 1986) is required as well as integration host factor (IHF). This factor is encoded by two genes and has been shown previously to be necessary in phage integration and excision (Eisenstein, 1987). The 314 bp sequence has conserved IHF binding sites. It is thought that IHF mediates the recombination between the inverted repeats (Dorman et al, 1987).

Phase variation may give an advantage to the invasive organism. It has been demontrated for *Proteus mirabilis* that fimbriae are virulence determinants at the colonization stage whereas they reduce virulence

at later stages of infection. The rate of phase variation is such that related strains may be of either phenotype. At the colonization stage, the Fim^+ strains are selected for and will propagate at a higher rate since these strains will adhere more effectively to host cells than Fim^-. At later stages, Fim^- strains generated by phase variation will be selected for as these are more likely to evade recognition by the host immune response. The presence of both phenotypes in genetically related strains is in this way favoured (Eisenstein, 1988).

A different mechanism to generate phase variation is found in *Bordetella pertussis*. In the promoter region of the fimbrial operon, there is a conserved sequence of cytosine residues (approximately 15). It has been found that Fim^- expression is due to deletions or additions of between 1 and 5 cytosine residues in this region (Willems et al, 1990). It is thought that transient misalignments of reiterated sequences during replication give rise to increased incidents of duplication and deletion mutations (Willems et al, 1988). The cytosine residues separate the activator binding region from the transcriptional initiation site. The distance between these regions, brought about by changes in the length of the polycytosine sequence, affects the efficiency of transcription (Willems et al, 1990).

A second mechanism involved in *B. pertussis* fimbrial regulation involves a positive control mechanism from the *trans*-acting locus, *bvg*. There are three polypeptides synthesized within the *bvg* locus that have been implicated in sensory transduction (Willems et al, 1988). A conserved base sequence near the 5' end of the *fim* locus appears to be the positive regulation binding site for a *bvg* gene product. Homologies have been shown between the *fim* promoter and the promoter for the

gene encoding the pertussis toxin also regulated by the *bvg* gene products (Willems et al, 1988). It is thought that these genes may be coregulated, further implicating fimbriae in pathogenesis. It is interesting to note that *bvg* has been shown to be transcribed in a temperature sensitive manner (Willems et al, 1988). Thus it appears that the positive regulatory mechanism may induce temperature sensitive expression of fimbriae as was the case in K88 fimbriae in *E. coli.*

Phase variation in *Moraxella bovis* involves switching between α-pilin and β-pilin fimbriae (Marrs et al, 1988). Phase transition occurs at one switch per ten thousand generations. Restriction mapping of the segment involved in the coding of these neighbouring loci indicated that the region responsible for the phase variation was a 2 kbp segment which was invertable (Marrs et al, 1988). This inversion places the promoter in front of either α-specific or β-specific sequences such that only one of these genes is expressed (Marrs et al, 1988). Comparisons of this segment and a 1 kbp inverted segment that controls phase variation in *Salmonella typhimurium* flagellae show homology at the ends of the invertable sequences. This 1 kb segment of *S. typhimurium* is known to contain a *hin* gene coding for a recombinase. A gene for a similar enzyme may be contained within the 2 kbp segment mediating the recombination of this fragment in *M. bovis* (Marrs et al, 1988).

As previously mentioned, organisms pathogenic to mammals must overcome active immunological defense mechanisms. In *Neisseria gonorrhoeae,* this is mediated in part through antigenic variation of fimbriae. As these structures are borne externally, they would be

expected to be immunogenic. The fimbrial subunit can be divided into a constant domain (C), at the N-terminus, a semivariable domain (SV) and a hypervariable domain (HV), at the C-terminus (Hagblom et al, 1985). The HV domain has conserved ends consisting of cysteine residues that form disulphide bridges. The variable regions are the immunodominant domains. For this reason, there is low immunological cross reactivity of fimbriae from different strains (Virji and Heckels, 1983).

Recombination between fimbrial pseudogenes and genes increase antigenic variation in *N. gonorrhoeae.* Silent loci such as *pil* S were found to show homology to the *pil*E1 and *pil*E2 fimbrial genes but do not possess a promotor sequence. These silent loci have 15 to 20 times the relative amount of variable domains. The *pil*E1 and *pil*E2 genes can recombine with silent loci in the HV and SV regions allowing for a wide range of antigenic variants (Saunders, 1985). By constantly recombining in this way *N. gonorrhoeae* is capable of changing its antigenicity to avoid the immune response of the host.

Bacterial fimbriae are widespread in gram negative bacteria. This implies a role for fimbriae which is conserved amongst members of this group. A number of these roles have been identified. These include conjugative DNA transfer and adherance to substrates in infection. For surface fibrils to be selected for, they must illicit only a minimal immunological response in their host. Fimbriae are regulated at both the level of transcription and the level of translation through a variety of mechanisms which enable bacteria to evade the host immune response while maintaining virulence.

Fungal Fimbriae

Surface proteinaceous fibrils morphologically similar to bacterial fimbriae and pili were observed on sporidial cells of the heterobasidiomycete *Ustilago violacea* by Poon and Day (1974). They reported that fibres were 1-10 μm in length with a constant diameter of 6-7 nm. They appeared to be curved, flexible, and unbranched. The number of fimbriae on the cell surface was found to be stage dependent as cells in the logarithmic phase of growth were far more fimbriated than stationary phase cells (Poon and Day, 1975). Freeze etched preparations showed fimbrial sized fibres on the outer surface of the plasmalemma and corresponding projections on the inner surface, suggesting that these fimbriae may be anchored in the cytoplasm.

Pronase and protease treatments degrade fimbriae (Poon and Day, 1974; Gardiner and Day, 1985). Other enzyme treatments were ineffective, indicating that these fibres are largely made up of protein. Dissociation of fimbriae from the cell surface may be accomplished by a variety of methods including: mechanical agitation, sonication and centrifugation in a high concentration sucrose gradient (Poon and Day, 1974). The purified fimbrial subunits have been shown to be proteinaceous using coomassie stain and silver stain procedures which are protein specific (Gardiner and Day, 1985).

Their morphology and proteinaceous constitution indicates that there are strong similarities between fungal and bacterial fimbriae and pili. The function, structure and genetic organization are discussed in the following sections.

Function

The morphological similarity of bacterial and fungal fimbriae is well established but this does not necessarily imply a similar functionality. Many roles attributed to bacterial fimbriae have been tested within the fungi.

Saccharomyces cerevisiae, an economically important ascomycetous yeast in the brewing industry, has been shown to possess fimbriae of a similar diameter but substantially shorter in length than those of *U. violacea* (Day et al, 1975). The role of fimbriae was assessed with respect to flocculation, an adhesive trait characteristic of various strains. Flocculent strains were found to be densely fimbriated whereas non-flocculant strains were sparsely fimbriated. Similarly, pronase treatments which remove fimbriae were found to inhibit flocculation. α-amylase which is known to not affect fimbriae (Poon and Day, 1974), also inhibits flocculation. Fimbriae may be a component of the mannan-protein complexes previously found to be necessary for flocculation (Day et al., 1975; Stewart et al, 1973). Thus, similar to their bacterial counterparts, fimbriae of *S. cerevisiae* are involved in adhesion.

In *Candida albicans*, a common parasite of humans, an adhesin associated with "extracelluar polymeric material" has been shown to be a virulence determinant in infections of mice (McCourtie and Douglas, 1984). Characterization of this adhesin indicates that it is a mannoprotein (McCourtie and Douglas, 1981). Proteolytic digestion of the adhesin rather than α-mannosidase digestion affects its ability to competitively inhibit adhesion of *C. albicans* to buccal epithelial cells (Critchley and Douglas, 1987a). Carbohydrates and lectins were used to

inhibit adhesion of *C. albicans* to epithelial cells. L-fucose and winged pea lectin, a fucose binding lectin, inhibit adhesion of a number of *C. albicans* strains whereas N-acetylglucosamine (N-AGA) and wheat germ agglutinin, a N-AGA binding lectin, were found to inhibit adhesion in the remaining strains (Critchley and Douglas, 1987b). There appear to be at least two extracellular, polymeric, proteinaceous adhesins, in *C. albicans*, differing in the specificity of carbohydrate binding.

The role of fimbriae in pathogenesis has been investigated for the mycoparasitic fungus *Piptocephalis virginiana* which parasitizes members of the Mucorales. Fimbriae have been confirmed on the surface of the host species and the protein subunits were shown to be recognized by a polyclonal fimbrial antiserum (AU) (Rghei et al, 1992). Preincubation of host and mycoparasite with AU decreased the level of contact between these organisms implying a role for host fimbriae. Inhibition in the level of contact would be expressed later as a decrease in virulence. *P. virginiana* appears to exploit the fimbriae of the host in order to establish a parasitic association.

The role of fimbriae in conjugation of *Ustilago violacea* was evaluated. Conjugation of *U. violacea* may be subdivided into two stages prior to genetic transfer: pairing of cells of opposite mating type and development of the conjugation tube (Day and Poon, 1975). It has become evident that factors which affect the presence of fimbriae similarly affect the ability of pairs to complete conjugation (Day and Poon, 1975a). These factors do not seem to affect the initial pairing, hence the role of fimbriae in conjugation may constitute more than simply adhesion.

Electronmicroscopic investigation of mating pairs indicate that *U.*

violacea pairs may remain separated by up to 20 μm during conjugation (Day, 1976). When cells in early stages of mating are suspended in water and examined microscopically, pairs are able to maintain a constant orientation with respect to one another and seem to rotate about the same axes. These observations cannot be attributed to fimbrial adhesion alone between preconjugative cells as the flexible nature of the fimbrial fibre could not support rotation (Day, 1976). Conjugation tubes may grow along the axis generated by the bound fimbriae allowing stability for genetic transfer to occur. Recently, a polyclonal antiserum (AU) raised against purified *U. violacea* fimbrial proteins have been shown to inhibit conjugation (Boulianne, 1990).

Fungal fimbriae seem to share both morphological and functional similarities with their bacterial counterparts. These fibrils appear to be required for adhesion, conjugation and virulence.

Subunit Structure

The structure of bacterial fimbriae and pili have been known for some time. The presence of both major and minor structural proteins as well as proteins involved in specific adhesion (Stromberg et al, 1990), length modulation (Baga et al, 1987; Maurer and Orndorf, 1987), structural specialization (Lindberg et al, 1987) and antigenic variation (Hagblom et al, 1985) has been shown for many fimbrial types.

Fungal fimbriae have been shown to consist mainly of protein as indicated by pronase (Poon and Day, 1975) and protease (Gardiner and Day, 1985) digestion. Dissociation of these surface fibrils by mechanical agitation has allowed for the characterization of the proteinaceous subunits. These have been shown to have a molecular

mass of 74 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Isoelectric focusing (IEF) and two dimensional gel electrophoresis (2-D) indicate that there are at least six isoforms (Castle et al, 1992).

Recently, the *U. violacea* fimbrial protein has been found to contain approximately 10% carbohydrate. Fimbrial proteins were found to stain with periodic acid Schiff reagent (PAS). Defimbriated *U. violacea* cells were allowed to reform fibrils in the presence of tunicamycin which inhibits glycosylation. A 67 kDa polypeptide which no longer stained with PAS reacted with AU (Castle et al., 1992). The 74 kDa protein of *U. violacea* is specifically bound by *Galanthus nivialis* agglutinin (GNA), a mannose binding lectin, but is not bound by lectins specific for three other carbohydrates (Castle et al, 1992). α-mannosidase-digested fimbrial protein is no longer bound by GNA and is approximately 10% smaller in terms of molecular mass than the 74 kDa protein (Castle et al, 1992). Thus, the fimbrial subunit of *U. violacea* is a mannoprotein consisting of approximately 10% carbohydrate.

Polyclonal antisera directed against both *U. violacea* (AU) and *Rhodoturula rubra* (AR) were derived from the corresponding 74 kDa fimbrial subunits. The sera were found to bind their own fimbrial subunits but not the other (Gardiner and Day, 1985). Numerous fungi from diverse taxonomic groups were tested for cross reactivity to each of these polyclonal antisera. The majority of yeast-like (Gardiner et al., 1982) and filamentous (Gardiner and Day, 1987) fungi were bound by one or both antisera indicating that at least some of the epitopic sites are conserved among various groups of fungi. Interestingly, members of two algal classes also shared some antigenic sites (Day et al.,

1986). Thus, fimbriae have been found to be widespread and conserved within the fungal kingdom and even beyond.

Conservation of antigenic sites has been used in order to assess differences in the fimbrial protein subunits of various fungi. SDS-PAGE separated on total soluble or purified fimbrial proteins of both filamentous and yeast-like species were immunoblotted and reacted with antiserum. Zygomycetous fungi, *Mortierella candalabrum*, *M. pusilla*, *Phascolomyces articulosis* and *Piptocephalis virginiana,* were shown to have proteins of 60 and 57 kDa, 64 kDa, 64 kDa, and 94 and 91 kDa, respectively, which share antigenic sites with the fimbrial protein of *U. violacea* (Rghei et al, 1992). All but *P. virginiana* were shown to possess fimbriae and the corresponding electroeluted proteins spontaneously formed fimbrial sized fibrils. The reason for the presence of antigen without detectable fimbriae in *P. virginiana* is uncertain.

The filamentous Basidiomycete, *Coprinus cinereus*, was shown to possess surface fibres. Western blot analysis of total soluble proteins yielded 37 and 39 kDa proteins with isoelectric points between 6.1 to 7.6 (Boulianne, 1990). The molecular mass and isoelectric points of these proteins differ from those of *U. violacea*. Electroeluted 37 and 39 kDa proteins of *C. cinereus* spontaneously formed fibrils of similar dimensions to those of native fimbriae. The elution profile of these proteins from a Sephadex G100 column suggested that the 37 kDa protein is polymeric and is thus the fimbrial subunit proper (Boulianne, 1990).

Fimbrial proteins appear to be similar in terms of antigenicity even between quite diverse taxa. However, there seems to be substantial

variation in the size and charge of these subunits amongst species.

Genetics

Much knowledge has been gained in recent years with respect to the organization of bacterial fimbrial gene clusters (Reigman et al, 1990), the role of gene products (Baga et al, 1987) and their regulation (Willems et al, 1990). Questions concerning the genetic organization of fungal fimbriae have not as yet been addressed in depth. However, there are a few points which should be mentioned.

Fimbrial regeneration has been examined in *U. violaceae*. Fimbriae were removed from sporidial cells of *U. violacea*. These cells were placed in cyclohexamide (an inhibitor of eukaryotic translation), chloramphenicol (an inhibitor of prokaryotic and mitochondrial translation) or rifampin (an inhibitor of transcription). Fimbriae were incapable of regeneration in the presence of either cyclohexamide or rifampin indicating that generation of mRNA from the nuclear genome and subsequent translation is required for fimbrial synthesis (Poon and Day, 1975). This differs from bacterial fimbriae as they have been shown to be synthesized from a pool of precursors following fimbrial removal.

Castle has generated a cDNA library from *U. violacea* mRNA extracted from log phase cells regenerating fimbriae (personal communication). The polyclonal antiserum was used to screen the library for production of polypeptides which share epitopes with the 74 kDa *U. violacea* fimbrial subunit. Three 1500 bp cDNA fragments (pfim 3-1, pfim 4-1 and pfim 6-1) were found to express fimbrial antigen. These were shown to be identical by restriction analysis and Southern hybridization (Castle, personal communication). Similarly, a fourth

cDNA fragment (pfim9-1) of 250bp in length was identified. This fragment did not hybridize to the other cDNAs. It was suggested that the different cDNAs may be derived from either nonoverlapping mRNA fragments or mRNAs coding antigenically related proteins. Since pfim3-1 is significantly larger than pfim9-1 and was obtained independantly 3 times from the amplified cDNA library, pfim3-1 was used preferentially for the initiation of studies on the organization, conservation and regulation of fungal fimbrial genes.

Materials and Methods

Fungal Cultures

Coprinus cinereus and *Schizophyllum commune* cultures were maintained through serial explants on *Coprinus* complete medium (CCM) and *Schizophyllum* complete medium (SCM) at room temperature. Liquid cultures were inoculated with hyphae that were fragmented in a Sorval Omnimixer at high speed for 1 minute with approximately 100 mL of liquid medium. The medium containing the fragmented hyphae was transfered to the culture medium and incubated at room temperature.

Monokaryotic *Coprinus cinereus* cultures 18632 (mating type A5B5, paba-2, nic-4) and 18633 (mating type A6B6, pdx) were mated by placing small agar plugs (2mm squares) containing hyphal fragments on CCM at a distance of approximately 1 cm. Dikaryon formation (C6323) was confirmed by the presence of clamp connections and by auxotrophic complementation on *Coprinus* minimal medium (CMM).

Monokaryotic prototrophic wild isolates 1-136, 1-137, 1-138 and 1-139 of *Schizophyllum commune* were obtained from Dr R. C. Ullrich from the University of Vermont. Dikaryotic cultures 1369 (1-136 x 1-139) and 1378 (1-137 x 1-138) were derived and confirmed by the presence of clamp connections.

Fungal Growth Media

CCM (Fries, 1953):

Glucose	20 g
L-aspargine	2.0 g
Coprinus Salt Solution	25.0 mL
Thiamin (10% stock(w/v))	1.0 mL
Yeast Extract	0.75 g
Peptone	0.75 g
Malt Extract	0.60 g

Brought to 1 L with dH_2O

Coprinus Salt Solution:

Ammonium Tartrate	20.0 g/L
KH_2PO_4	40.0 g/L
Na_2SO_4	90.0 g/L
$Na_2SO_4 \cdot 10H_2O$	11.2 g/L

CMM (Fries, 1953):

Asparagine	1.0 g
$MgSO_4 \cdot 7H_2O$	0.06 g
NH_4Cl	0.75 g
$Na_2HPO_4 \cdot 7H_2O$	1.37 g
KH_2PO_4	0.68 g
Na_2SO_4	0.15 g

SCM (Snider and Raper, 1958):

$MgSO_4$	0.5 g
KH_2PO_4	0.46 g
K_2HPO_4	1.0 g
Peptone	2.0 g
Yeast Extract	2.0 g
Glucose	20.0 g

Brought to 1 L with dH_2O

Protein Characterization

Protein Extraction

Total soluble protein (TSP) was obtained from both *C. cinereus* and *S. commune* with the same procedure. Mycelia were grown in 500 mL liquid cultures at room temperature. Mycelia were collected by filtration through cheesecloth followed by several rinses with distilled water. Mycelia were ground in an equal volume of TEPI [10 mM Tris, 1 mM ethylenediaminetetra-acetic acid (EDTA), 1 μM phenylmethylsulfonyl fluoride and 1 mM iodoacetamide, pH 6.8] and 2 volumes of either silica gel or 100 mesh glass beads in a chilled mortar and pestel. The mixture was centrifuged 10 minutes at 10 000

x g at 4°C. The supernatant was twice mixed with an equal volume of n-butanol followed by centrifugation at 1000 x g for 10 minutes at 4°C. The lower aqueous phase was removed and dialyzed against TE (10mM Tris pH 8.0 and 1 mM EDTA) at 4°C for a minimum of 2 hours followed by dialysis against gdH_2O for 30 minutes. Dialized samples were lyophilized, resuspended in a minimal volume of TEPI and stored at -20°C.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

The procedure of Laemlli (1970) was used for sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were separated on an 11% separating gel and a 4% stacking gel with the Biorad Protean II minigel apparatus. The gel mixture (0.375 M Tris-Cl (pH 8.8), 10.8% acrylamide, 0.3% N,N'-methylenebisacrylamide (BIS), 0.1 % Sodium dodecyl sulphate (SDS) was degassed prior to the addition of 0.05% N,N,N',N'-tetramethylenediamine (TEMED) and 0.05% ammonium persulphate (APS)) . Gels of 0.75 mm thickness were generated. The stacking gel mixture consisted of 0.125 M Tris-Cl (pH 6.8), 3.8% acrylamide, 0.1% BIS, 0.1% SDS, 0.1% TEMED and 0.05% APS.

Protein samples were added to an equal volume of sample buffer (125mM Tris-Cl (pH6.8), 20% glycerol (v/v), 4% SDS (w/v), 10% 2-mercaptoethanol and 0.01 % bromophenol blue). Protein samples were then heated to 95° C for 2 minutes and 25 µL was loaded onto the gel. The running buffer (pH 8.3) used was 0.3 % Tris, 1.44% glycine and 0.1% SDS. Gels were run at between 150 and 200 volts (Constant voltage). Gels were stained, transfered to nitrocellulose or used to excise specific polypeptides.

Proteins were stained with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid for a minimum of 1 hour. The gels were destained in 40% methanol and 10% acetic acid until the desired background was obtained.

Two Dimensional Gel Electrophoresis

Two dimensional gel electrophoresis was performed with the methods of Tempst and coworkers (personal communication). Protein samples in TEPI were lyophilized and resuspended in sample buffer (9 M urea, 4% NP-40, 2% ampholytes and 1% dithiothreitol (DTT) (w/v)).

The isoelectric focusing gel solution consisted of 9M urea, 2% ampholytes, 3.13% acrylamide (w/v), 0.19% BIS (w/v), 0.07% ammonium persulphate (w/v) and 0.07% TEMED (v/v). The gel was allowed to polymerize inside the tubes (10 cm long, 1 mm inner diameter) for 1 hour.

The upper electrophoresis solution was 0.02 N NaOH and the lower solution was 0.01 N H_3PO_4. The gels were focussed at 400 volts for 1 hour to allow the current to approach 0 mA. The samples were loaded with a Hamilton syringe onto the upper surface of the gel and were separated for 17 hours at 800 volts.

The gels were extruded and allowed to equilibrate in buffer (10% glycerol, 8.6 mM DTT, 2% SDS, 0.01% m-cresol green and 0.125M Tris) for 5 minutes with mild agitation. The tubes were either laid directly over 11% SDS-PAGE gels or stored at -20^{o}C.

Immunodetection of Fimbrial Antigen

Proteins from SDS-PAGE gels were transfered to nitrocellulose

membranes (BioRad Transblot, 0.45 μm pore) in a Transblot apparatus (Biorad). Proteins were transfered overnight in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol (v/v)(pH 8.3)) (Towbin et al., 1979) at constant voltage (30 V). The voltage was increased to 60 V for 60 minutes. The membrane was rinsed in Tris buffered saline (TBS)(20 mM Tris and 500mM NaCl (pH 7.5)). The membrane was placed in 3% gelatin in TBS for a minimum of 2 h then rinsed in TTBS (0.05% tween-20 in TBS).

A polyclonal antiserum raised against *U. violacea* fimbrial protein was used to detect fimbrial antigen. The antiserum was partially purified by protein-A sepharose chromatography prior to use. A 1/1000 dilution of antiserum was incubated with the membrane for 2 h in 1% gelatin in TTBS. The membranes were vigorously shaken in 3 changes of TTBS for a total of 90 minutes. Goat-anti-rabbit IgG (1/15000 dilution in 1% gelatin and TTBS) with an alkaline phosphatase conjugate (Promega) was incubated with the membrane for a period of 1 h. Three vigorous washes in TTBS followed by a wash in TBS were performed. The membrane was immersed in colour reagent (0.1 mM Tris (pH 9.5), 1 mM $MgSO_4$, 0.03% nitroblue tetrazolium and 0.015% biochloroindolyl-phosphate) overnight in the dark. Membranes were rinsed in distilled water and were allowed to air dry on filter paper.

Nucleic Acid Characterization

Fungal DNA Isolation

Isolation of fungal DNA was performed with the protocol of Murray and Thompson (reviewed in Taylor and Natvig, 1987). Mycelia were filtered through cheesecloth and rinsed with 0.25 M EDTA. The entire

cheesecloth preparation was placed in liquid nitrogen and freeze dried.

The lyophilized cells were ground in the presence of liquid nitrogen. The DNA was extracted in 12 mL/g (dry weight of mycelia) high salt hexadecyl trimethylammonium bromide(CTAB) solution (1% CTAB, 0.05M Tris-Cl (pH 8), 0.7M NaCl and 10mM 2-mercaptoethanol) at 55^{o}C for 30 minutes. One volume of chloroform: isoamyl alcohol (24:1) was added, mixed by inversion and centrifuged in Corex tubes at 13000xg for 10 minutes at room temperature. The upper aqueous phase was mixed with 2ml of 10%CTAB and 0.7 M NaCl. An equal volume of chloroform: isoamyl alcohol was added and the resulting mixture was centrifuged at 13000xg. The aqueous phase was added to an equal volume of low salt CTAB buffer (1%CTAB, 0.05M Tris-Cl (pH 8) and 10mM EDTA). DNA was allowed to precipitate at room temperature for 30 minutes and was collected by centifugation at 2000 x g for 5 minutes.

The pellet was suspended in CsCl in TE to a final density of 1.69 g/mL with 250µg bis-benzamide. The suspension was centrifuged at 61000 rpm for 22 hours in a Beckman TL-100 microultracentrifuge. DNA was visualized under ultraviolet (UV) illumination and drawn off with an 18 gauge syringe. The samples were dialyzed against TE for 2 h. The dialyzed samples were divided into 400µL samples to which 100µL ammonium acetate (7.5M stock) and 1 mL ethanol were added. DNA was precipitated at -20^{o}C and pelleted in a microcentrifuge. The pellet was washed in 70% ethanol, dried and resuspended in TE. DNA purity and concentration were assessed with a Beckman DU-50 spectrophotometer.

Fungal DNA miniprep

A microcentifuge tube was half filled with ground lyophilized cells and 500 µL of extraction buffer (10mM EDTA, 10mM Tris, 0.5% SDS, 5mg/mL pronase) was added (Haj-Ahmad, personal communication). The mixture was incubated at 37°C for 30 min and was mixed occasionally by inversion. Following 5 min centrifugation, the supernatant was twice extracted with an equal volume of phenol: chloroform: isoamyl (25:24:1). 200 µL of 7.5 M ammonium acetate was added to the supernatant. 2.5 volumes ethanol was added and mixed. The sample was placed at -20°C for 15 minutes and the DNA was pelleted by centrifugation for 10 min at 4°C. The pellet was washed in 70% ethanol, dried and resuspended in TE.

Restriction Endonuclease Digests

Restriction enzymes (Boeringer-Mannheim) were used at an concentration of 0.5 units/µL. The amount of DNA varied with the size of the molecule being digested; between 50 and 250 ng DNA. The sample and enzyme were suspended in a total volume of 20µL with the buffer specified by the manufacturer for the enzyme. The samples were incubated at 37oC for a minimum of 2 h for complete digestion.

Agarose Gel Electrophoresis

Restriction enzyme digested DNA was analysed by electrophoresis in 0.55% to 1.0% agarose (w/v) gels. Electrophoresis buffer was TAE (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.0). To the DNA samples was added 1/10 volume of loading buffer (0.25% bromophenol blue and 20% glycerol in TAE) and samples were heated to 65°C for 2 min. The

samples were loaded on the gel and a constant voltage was applied (15-75 volts). Gels were stained in ethidium bromide (1μg/mL) for 20-40 minutes. The gels were then photographed under UV light to visualize the DNA.

Alkaline Southern Blotting

Photographed agarose gels were placed in 0.25 N HCl for 15 min at room temperature in order to acid nick the DNA. The gels were placed on a filter paper wick with ends emmersed in of 0.4 N NaOH. A nylon membrane (ICN Biotrans) was placed directly on top the gel and several layers of absorbant paper were stacked on top. DNA was eluted from the gel to the membrane for a minimum of 4 h. The nylon membrane was neutralized for less than a minute in a small volume of SSC (150mM NaCl, 15mM $Na_3C_6H_5O_7 \cdot 2H_2O$ pH 7.0). DNA was cross linked to the membrane with UV light in a Statagene crosslinker. The membrane was then allowed to air dry on filter paper.

Labelling Reactions

Radioactive probes were generated using the Pharmacia Oligolabelling kit by the random priming method of Feinberg and Vogelstein (1983). Up to 75ng of DNA was boiled for 10 minutes. To this was added 5 μL of the random hexamer mix and immediately placed on ice. Label, 30 μCi α-^{32}P dCTP (ICN), and 5 units Klenow fragment of DNA polymerase I were added and the mix was incubated at 37°C for 2 to 5 h. Incorporation of label into polynucleotides was determined by the ratio of labelled 10% trichloroacetic acid precipitated to unprecipitated radioactivity as measured by scintillation counting.

Typically values ranged between 25 and 50% incorporation.

Hybridization of Radiolabelled DNA to RNA and Plasmid DNA

Hybridization of ^{32}P labelled DNA was according to the procedure of Mahmoudi and Lin (1989). Hybridization solution [0.5 M NaPO4, 1% bovine serum albumen (BSA), 7% SDS, 0.5 M EDTA and 100 µg/mL herring or salmon sperm DNA] was added to the nylon membranes in a sealed bag for 30 minutes at 68°C prior to adding labelled DNA. Radioactive DNA (10^5 to 10^6 cpm/mL of hybridization solution) was denatured by heating to 100°C for 10 min. The denatured DNA was then placed in the hybridization solution and the bag was resealed. The bag was incubated at 68°C overnight, shaking gently.

Following hybridization, the membranes were removed and rinsed in 2x SSC and 0.1% SDS for fifteen minutes at 68°C. The washed membranes were placed on moist filter paper, sealed in hybridization bags and exposed to X-ray film (Kodak X-OMAT) with a Cronex intensifying screen for 4 to 96 hrs at -20° C.

Hybridization of Radiolabelled DNA to Fungal DNA

Hybridization of ^{32}P labelled DNA was performed by the protocol as described in Sambrouk and Coworkers (1989). Blots were incubated in prehybridization solution [2xSSC, 10% (w:v) dextran sulfate, 1% (w:v) SDS and 5x Denhardt's solution] for 30 min. at 65°C in a sealed bag. Radiolabelled DNA (105 to 106 cpm/mL of hybridization solution) was denatured for 10 min at 100°C with 150 µg/mL herringsperm DNA. The denatured DNAs were added to the prehybridization mix and resealed. The mix was incubated at 65°C for 16 to 20 h.

Library Construction

CsCl purified *Coprinus cinereus* DNA was digested partially for 5 min with *Sau* 3A restriction endonuclease in order to generate average fragment lengths greater than 10 kbp. Restriction fragments were ligated (Boeringer Mannheim T4 ligase) to bacteriophage λ (λGEM-11 *Bam* H1 arms cloning system vector, Promega Biotech). The ligation mix included 1μg digested DNA, 0.5 μg vector DNA and 1 unit T4 ligase (Boeringer-Mannheim). Ligation was completed at 25°C for 2 hours. The ligation mix was transfered to a phage capsid mix (Packagene Promega) and allowed to sit for 2 hours at room temperature. 500 μL SM (0.58% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, .05 M Tris and 0.01% gelatin (pH7.5)) and 25 μL of chloroform were added. The library was stored at 4° C.

Library titer was determined by plating 100μL of serially diluted library on Luria-Bertani broth (LB) (1% bacto-tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0) with 10 mM $MgSO_4$ (LB-Mg). Diluted phage were preincubated at 37°C for 15 minutes with *E. coli* strain LE 392 (130 μL of 1 unit OD_{600} culture) cells prior to addition of top agarose (0.7% agarose in LB-Mg). The plates were incubated at 37°C overnight. The number of placques were counted and the number of placque forming units (pfu) in the original phage suspension was estimated.

Library Screening

The *Coprinus cinereus* library was screened by plaque hybridization with ^{32}P labelled fimbrial cDNAs derived from *Ustilago violacea*, these plasmid clones are referred to as pfim 3-1 and pfim 9-1 (Castle, personal communication). The screening process involved three separate stages for confirmation. Library was plated with LE 392 such

that approximately 2500 plaques per plate were generated. A total of 12500 plaques were screened in order to be 99% confident of obtaining a recombinant phage with sequence homologous to a fimbrial cDNA ($P = 1-(1-A)^N$ where P is the probability of obtaining the recombinant, A is the relative abundance of a single clone with respect to the size of the genome ($4x10^4$) and N is the number of plaques screened) (Frischauf, 1987).

Nylon membranes were laid over the plates until thoroughly wetted. The membranes were removed, wrapped in aluminium foil and autoclaved on the dry cycle for 2 minutes (121°C, 22 PSI). The membranes were allowed to air dry. Membranes were hybridized with labelled cDNA as outlined above. Both pfim3-1 and pfim9-1 were used in separate hybridizations. After hybridization and analysis with one cDNA, the bound radiolabelled DNA was stripped from the membrane by pouring two 250 mL aliquots of boiling 1% SDS, 0.1 x SSC. The hybridization procedure was then repeated with the second cDNA.

Hybridized membranes were exposed to X-ray film. The developed X-ray film was then used to locate the plaques that corresponded to the hybridized clones. These were transfered to 200 µL SM buffer and stored at 4°C.

A lawn of LE 392 was plated on LB-Mg with 0.7% top agarose and allowed to solidify. One µL of suspended phage from the first hybridization analysis was spotted on the surface of the lawn. These were placed at 37°C, overnight. Hybridization analysis with the cDNA probes was repeated and the appropriate plaques were again purified.

The final screen was performed by plating 10 µL of suspended phage per plate. Phage were lifted with nylon membranes from plates

incubated overnight. Hybridization was again performed. Clones that showed widespread hybridization to plaques were considered to share homology with the pfim cDNAs. A single clone, C2-3A, which showed strong binding to pfim3-1 was used for most subsequent procedures.

Lambda DNA Extraction

Clones were grown in 100 mL shake cultures of LE 392 overnight at 37°C. DNA was isolated by a modified method of Manfioletti and Schneider (1988). Chloroform was added (1.5 mL) to the culture flask and the flask was shaken for 30 minutes. The chloroform was allowed to settle and the aqueous portion was removed.

1.2 mg DNAse I was added to 40 mL of the phage infected bacterial culture culture (the remaining 60 mL was stored at 4°C as a stock lysate). This was allowed to stand 15 minutes at room temperature. The mixture was centrifuged at 8000 x g for 10 minutes. 1.6 mL 0.5 M EDTA (pH 8), 2 mg proteinase K were incubated with the supernatant for 15 minutes. An equal volume of 10% CTAB and 1M NaCl was added and incubated at 65°C for 3 minutes. The mixture was cooled on ice for 5 minutes and centrifuged at 8000 x g for 10 minutes at room temperature. The pellet was dissolved in 4mL (1/10 original volume) 1.2 M NaCl.

The mixture was separated equally into microcentrifuge tubes and 2 volumes of ethanol was added. DNA was precipitated at -20° C for 30 minutes. The DNA was pelleted in a microcentifuge, rinsed with 70% ethanol, vacuum dried in a Savant SpeedVac Concentrator and resuspended in a minimal volume of TE.

Subcloning Into Plasmid Vector pUC 19

The inverted symmetrical lambdaGEM-11 multiple cloning site allowed the excision of insert DNA with the restriction endonuclease *Sac* I. The phage clone, C2-3A, insert was found to have 2 *Sac* I restriction sites. Equimolar amounts of C2-3A and pUC 19 were *Sac* I digested, mixed and ligated in order to subclone the three insert restriction fragments. The ligation mixture was then transformed into competent *E. coli* cells.

E. coli strain DH5α was grown overnight in 2 mL LB cultures. Half millilitre aliquots were used as inoculum for 50 mL cultures. These were allowed to grow for 2-3 hours (until OD_{600} = 0.5-0.6). Cells were collected by centrifugation and suspended in 25 mL of transformation buffer (5mM Tris and 75 mM $CaCl_2$ (pH 7.5)). Cells were left in transformation buffer on ice for a minimum of 6 hours, collected by cetrifugation and resuspended in 1 mL of transformation buffer. The competent cells were stored on ice for short periods of time (up to 24 h) or at -70^{o} C in 15% glycerol for longer periods.

For transformation, 100 µL of competent cells was added to up to 10 µL of a plasmid ligation mix (5ng DNA) and placed on ice for 30 minutes. The cells were then heat-shocked for 45 seconds at 42^{o}C and immediately placed on ice for 2 minutes. 900 µL SOC (2% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 20mM $MgSO_4$ and 20 mM glucose) was added. This mixture was incubated for 45 minutes at 37^{o}C. 100 µL samples were plated on LB-Amp-Xgal plates (LB plates with 40 mg/mL ampicillin and 800 µg 5-bromo-4-chloro-3-indolyl ß-D-galactoside (X-GAL)). The plates were incubated at 37^{o}C

overnight. Colonies with recombinant plasmids were detected by a-complementation of ß-galactosidase activity (Ullman et al., 1967). White colonies were transfered to 2 mL cultures of LB-Amp and grown overnight for plasmid DNA isolation.

Miniprep Plasmid DNA Isolation

2 mL cultures of transformed colonies were used for plasmid isolation by the method of Birnboim and Doly (1979). Microcentifuge tubes were filled with 1.5 mL of overnight cultures. These were centrifuged for 10 minutes. Cell pellets were suspended in 100 µL of lysing buffer (0.01 M Tris, 0.01 M EDTA and 4% SDS) with 5µg/mL of lysozyme and incubated on ice for 30 minutes. 200 µL of alkaline SDS (0.2 N NaOH and 1% SDS) was added and the mixture was incubated for 15 minutes at room temperature. Sodium acetate (150 µL of 3 M pH 4.8)was added and the mixture was incubated on ice for 1/2 hr. The mixture was centrifuged and the supernatant was transfered to clean microcentrifuge tubes. DNA was precipitated by addition of 1mL of absolute ethanol and left at -20^{o}C for 15 minutes. DNA was collected by centrifugation, washed with 70% ethanol, vacuum dried and resuspended in TE.

Isolation of DNA Fragments From Agarose Gels

DNA was separated by electrophoresis through agarose gels and was visualized with ethidium bromide staining. Gel pieces containing DNA fragments of interest were excised with a razor blade and transfered to microcentrifuge tubes. 100 µL phenol pH 8 was vigourously mixed with the gel fragment using a vortex mixer. The mixture was frozen at

-70°C for 15 minutes and centrifuged. The supernatant was extracted twice with phenol. 15 µL of 5M NaCL was added and DNA was precipitated in 70% ethanol at -70°C for 15 min. The DNA was pelleted by centifugation, dried and resuspended in TE.

Large Scale Plasmid Extraction

Transformed cells were grown in 250 mL LB-Amp. The culture was centifuged at 4000 x g for 15 min. The pellet was suspended in 6mL 25mM Tris, 10mM EDTA, 15% sucrose and 12 mg lysozyme (pH 7.5). The mixture was incubated on ice for 20 min followed by 10 min with addition of 12 mL of 0.2 M NaOH and 1% SDS. 7.5 mL of 3 M sodium acetate (pH 4.5) was added and incubated for 20 min on ice.

The mixture was transfered into 30 mL Corex centrifuge tubes and centrifuged at 10000 x g for 15 min. 50µg RNase A was added to the supernatant. RNA was digested at 37°C for 20 min. The aqueous mixture was extracted twice with phenol chloroform (1:1) with a 10 min centrifugation at 4000 x g separating the phases. The DNA was precipitated in 70% ethanol for 15 min at -20°C. The DNA was pelleted at 8 000 x g for 15 min at 4°C. The pellet was resuspended in 0.5 mL gd H_2O and aliquoted into microcentrifuge tubes. The DNA was precipitated, pelleted, dried and resuspended in a minimum volume of TE.

DNA Sequence Determinations

DNA was sequenced with the dideoxy nucleotide chain termination method of Sanger (1977) using the Sequenase Version 2.0 kit (United States Biochemical). Plasmid DNA (>5 µg) was alkaline denatured in 0.2 M NaOH and 0.2 mM EDTA for 30 min at 37°C. The mixture was

neutralized in 0.3 M sodium acetate (pH 4.5). DNA was precipitated in 70% ethanol and pelleted by centifugation. The pellet was washed in 70% ethanol, pelleted, dried and resuspended in a minimal volume of gdH_2O. The DNA concentration was assessed by the absorbance at 260 nm.

Oligonucleotide primers (16-mer) were annealed to complementary sequences near the multiple cloning site of pUC 19. Between 3 and 5 μg of template was added to 2 μL reaction buffer and 0.5-1.0 pmoles primer. The volume was adjusted to 10 μL. This mixture was heated to 65°C for 2 min then allowed to cool to room temperature for 15 to 30 min. Annealed primer and template were cooled on ice prior to extention (labelling) and termination reactions.

DNA was labelled by bringing the entire annealing mix to a final concentration of 6μM DTT, 0.2 μM of each dNTP (dGTP, dCTP, dTTP and ^{35}S-dATP) and 2 units polymerase enzyme (Sequenase Version 2.0) in a total volume of 15.5 μL. The reaction proceeded at room temperature for 2-5 min.

The termination reaction was performed at 37°C for 5 min. 3.5 μL samples of a label reaction was placed in each of four tubes containing 2.5 μL of 8 μM of one dideoxy nucleotide (ddNTP), 80 μM of each dNTP and 50 mM NaCl. To these reactions was added 4 μL of stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue and 0.05% Xylene Cyanol FF).

DNA Sequence Gels

Gel solution (6M urea, 3.7% acrylamide, 0.2% bis-acrylamide in TBE (90mM Tris, 90mM Borate and 2mM EDTA)) was degassed under vacuum

for 15 minutes prior to addition of 0.02% APS and 0.02% TEMED. A 10 mL aliquot was poured and allowed to polymerize (1 h) in order to seal the bottom of the gel apparatus. The remainder of the gel solution (90-100 mL) was poured and a straight edged comb was used to generate a uniform interface for the upper surface of the gel. The gel was allowed to polymerize in a horizontal position for 1 h prior to removal of the comb.

DNA was heated to 75°C for 2 minutes immediately prior to loading. The samples (2.5 μL) were loaded into sharktooth comb wells on the upper gel surface. Electrophoresis was performed at 1800 volts (constant voltage) for between 2 and 9 hours in TBE buffer.

The gel was removed from the surface of the glass plates by adsorption to Whatman paper (#3). The gel was overlaid with plastic film (Dow Chemical), vacuum dried (Bio-Rad Model 583 Gel Dryer) and exposed to X-Ray film (Kodak X-OMAT) for between 36 and 72 h.

RNA Extraction

All equipment and solutions used for RNA extraction were pretreated with diethylpyrocarbonate (DEPC) to inhibit RNAase activity. RNA was extracted from 0.1 gm ground lyophilized cells in 1 mL 6M guanidium hydrochloride (GuHCl), 0.02 M sodium acetate and 10 mM dithiothreitol, at room temperature for 30 min. The cell debris was pelleted in a microcentrifuge for 10 min. The supernatant was layered onto a 0.5 mL CsCl cushion (0.96 g/mL CsCl in 100mM EDTA). RNA was pelleted at 30 000 RPM for 15 h in a TLS-55 swinging bucket rotor in the Beckman TL-100 micro-ultracentrifuge. The centifuge tube was inverted on absorbant paper and the pellet was resuspended in chilled

DEPC treated water (DEPC-H_2O) on ice for 1 hour. The sample was transfered to a microcentrifuge tube and 35 µL 3 M sodium acetate (pH 4.5) was added. RNA was pelleted with 2.5 volumes ethanol at -20°C for 30 min followed by centrifugation at 4°C for 10 min and a rinse in 70% ethanol. The pellet was resuspended in DEPC-H_2O.

Formaldehyde Agarose Gels

RNA was separated at 50-60 volts (constant voltage) for 3-4 hours in 0.8% agarose and 2.2 M formaldehyde in running buffer (10 mM NaPO4 (pH7.0), 1mM EDTA, 5mM sodium acetate)(Rave et al., 1979). RNA was transfered to nylon membrane (ICN Biotrans) in 6x SSPE(900 mM NaCl, 60 mM NaH_2PO_4 (pH 7.4)) by capillary action as described for DNA transfer. RNA was cross linked to the membrane (stratagene UV crosslinker) and hybridized with ^{32}P labelled DNA. Hybridization was visualized by exposing the nylon membrane to X-ray film (Kodak X-omat) for 24 to 72 hours.

Results

Immunodetection of Polypeptides with Fimbrial Epitopes

Total soluble proteins (TSP) were extracted from *C. cinereus* and *S. commune*. Indirect immunodetection of fimbrial antigen was performed using a polyclonal antiserum (AU) raised against the fimbrial proteins of *U. violacea*. The polypeptides were separated by SDS-PAGE and were either visualized with Coomassie brilliant blue R-250 or transferred to nitrocellulose membranes and incubated with antiserum. As controls for the specificity of the antiserum, duplicate membranes were incubated with preimmune serum (NS) and the secondary antiserum, goat-anti-rabbit IgG, or secondary antiserum alone. The antiserum (AU) recognized two polypeptides with masses of 37 ± 7 and 39 ± 7 kDa from *C. cinereus* strain 18632 (figure 1) and a single polypeptide of 50 ± 2 kDa from *S. commune* (figure 1) strains 1378 and 1-136. NS treatment (figure 1) did not recognize any proteins.

Detection of proteins sharing epitopes with the fimbrial proteins of *U. violacea* had not been shown for *S. commune* prior to this study. A survey of the characteristics of the fimbrial antigen was made for comparison to other fimbrial proteins. TSP were separated by 2-D gel electrophoresis, transfered to nitrocellulose filters and fimbrial proteins were detected with antiserum, AU. At least 3 polypeptides were resolved at 50 kDa with isoelectric points ranging between 5.7 and 5.9 (figure 2).

Figure 1. **Fimbrial proteins of *C. cinereus* and *S. commune*.**

SDS-PAGE separation of total soluble proteins of *C. cinereus* (A) and *S. commune* (B). In both A and B, proteins in lane 1 are stained with Coomassie Brilliant Blue R250. Immunoblots in lane 2 were incubated with polyclonal antiserum raised against the fimbrial proteins of *U. violacea* followed by alkaline phosphatase (AP) conjugated goat anti-rabbit IgG and chromogenic substrate. Immunoblots in lanes A3 and B3 were incubated with rabbit preimmune serum followed by the AP-conjugated goat anti-rabbit IgG. The molecular masses of cross reacting polypeptides are indicated in kDa at the left side of the blots.

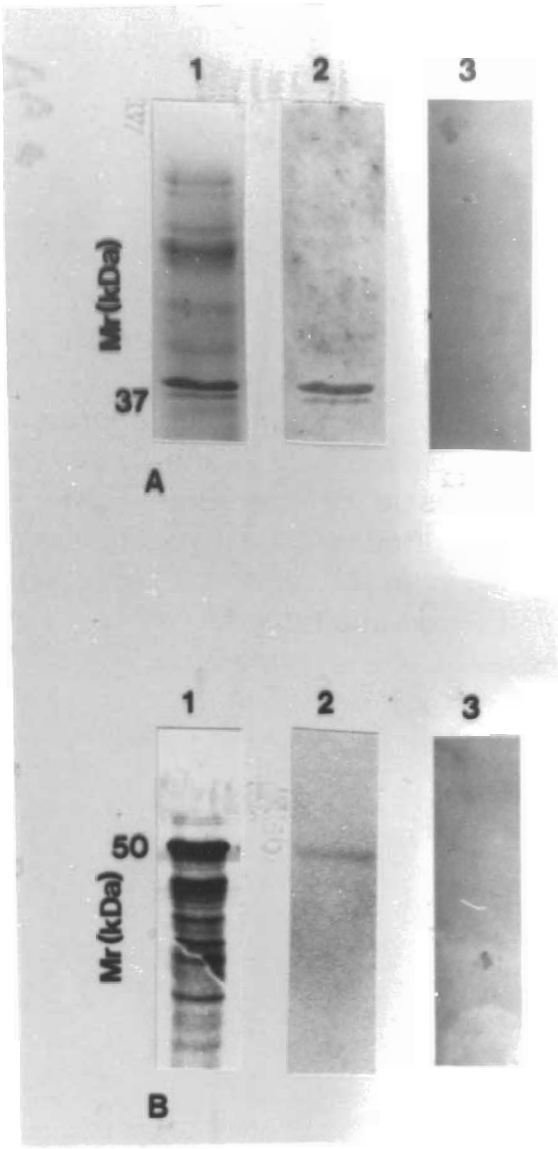
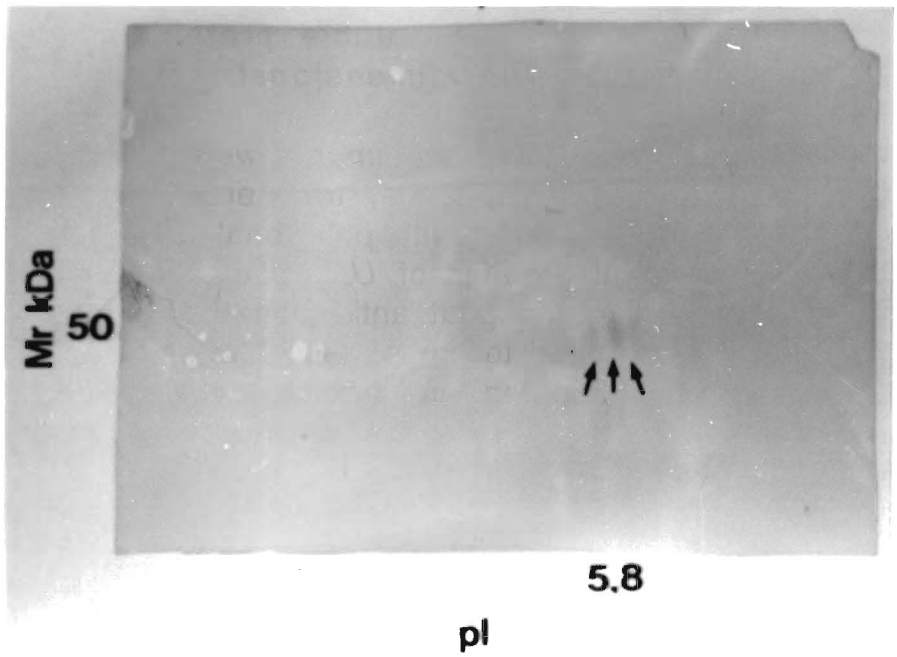

1
2
3
Mr(kDa)
37
A
1
2
3
50
Mr(kDa)
B

Figure 2. **Immunoblot of total soluble proteins of *S. commune* separated by two dimensional gel electrophoresis.**

Total soluble proteins of *S. commune* were separated by two dimensional gel electrophoresis, transfered to nitrocellulose membranes and incubated with polyclonal antiserum raised against the fimbrial proteins of *U. violacea* followed by alkaline phosphatase conjugated goat anti-rabbit IgG. Polypeptides of 5.6, 5.8 and 5.8 were found to cross react. Cross reacting polypeptides are indicated with arrows. The molecular mass is indicated in kDa to the left and the isoelectric point is indicated below.


Mr kDa
50
5.8
pI

Heterologous Hybridization of Fimbrial cDNAs to DNA of *C. cinereus* and *S. commune*

DNA from *S. commune* and *C. cinereus* was cut with the restriction endonucleases, *Hin* dIII and *Bam* HI. Hybridization of radiolabelled cDNAs from *U. violacea* indicated that sequences homologous to fimbrial transcripts were present in both these species (figure 3). Heterologous hybridization of cDNAs could thus be used in order to isolate DNA homologous to the fimbrial gene of *U. violacea*.

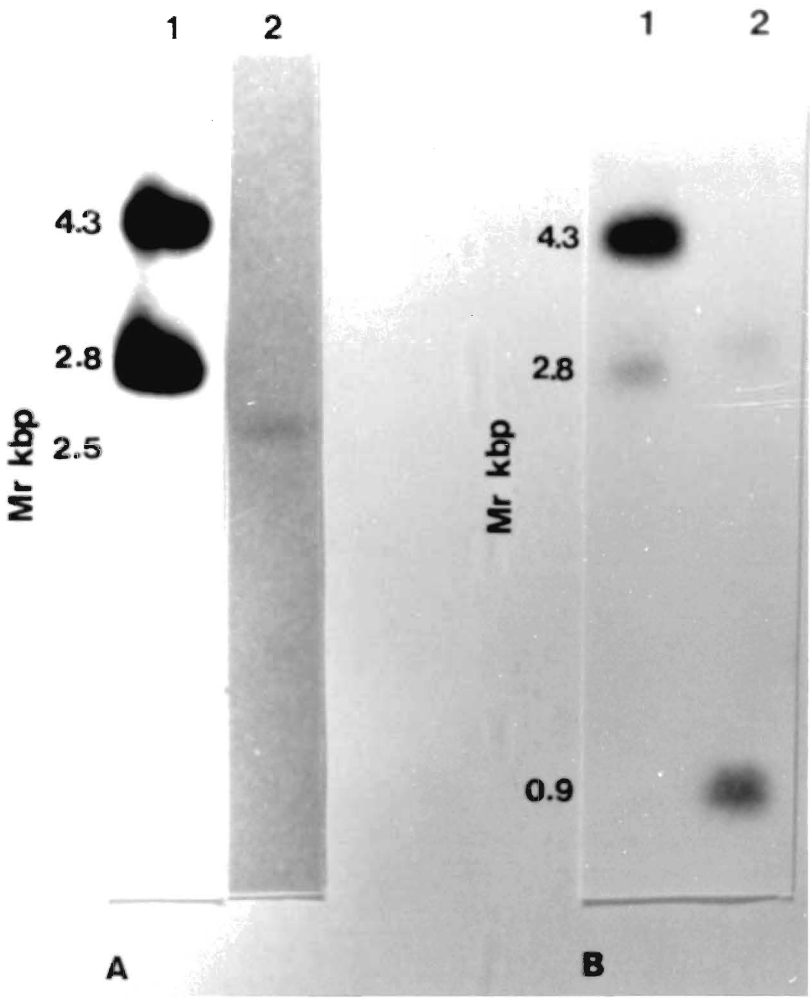
Library Construction and Screening for Homology to cDNAs

CsCl purified *Coprinus cinereus* genomic DNA was partially digested with the restriction endonuclease *Sau* 3A in order to generate termini compatible with the *Bam* HI restriction sites in the λGEM-11 cloning vector (Promega) (figure 4). Only fragments between 8 and 20 kbp in length could be inserted due to packaging constraints of the bacteriophage. Test digestions were made for 1, 3 and 5 min in order to determine the optimum reaction conditions to generate fragments between 8 and 20 kbp in length. DNA fragments were separated by agarose gel electrophoresis (figure 5). Five min digestions were found to generate the distribution of size fragment sizes optimal for cloning.

A library was generated with a titer of $5x10^5$ plaque forming units (pfu) per mL. Based on the estimated size of the fungal genome ($3.75 x10^7$ bp) (Dutta, 1974) and the average insert size (14 000 bp), 12500 plaques had to be screened such that there was 99% certainty of obtaining a single sequence with homology to the fimbrial cDNA (see materials and methods). 2500 pfu were plated with *E. coli* strain LE

Figure 3. **Heterologous hybridization of pfim3-1 to DNA from *C. cinereus* and *S. commune*.**

DNA from *C. cinereus* (A) and *S. commune* (B) was restriction digested with *Hin* dIII (1) and *Bam* HI (2). DNA fragments were separated by agarose gel electrophoresis, transfered to nylon membranes and hybridized with radiolabelled pfim3-1. The molecular masses of the hybridizing DNA fragments are indicated in kbp to the left of the blots. The size of restriction fragments were estimated from the relative migration of *Hin* dIII I DNA fragments as measured on ethidium bromide stained gels prior to DNA transfer. Size estimates of restriction fragments for all further Southern blots were determined similarly.


1
2
4.3
2.8
2.5
Mr kbp
A
1
2
4.3
2.8
Mr kbp
0.9
B

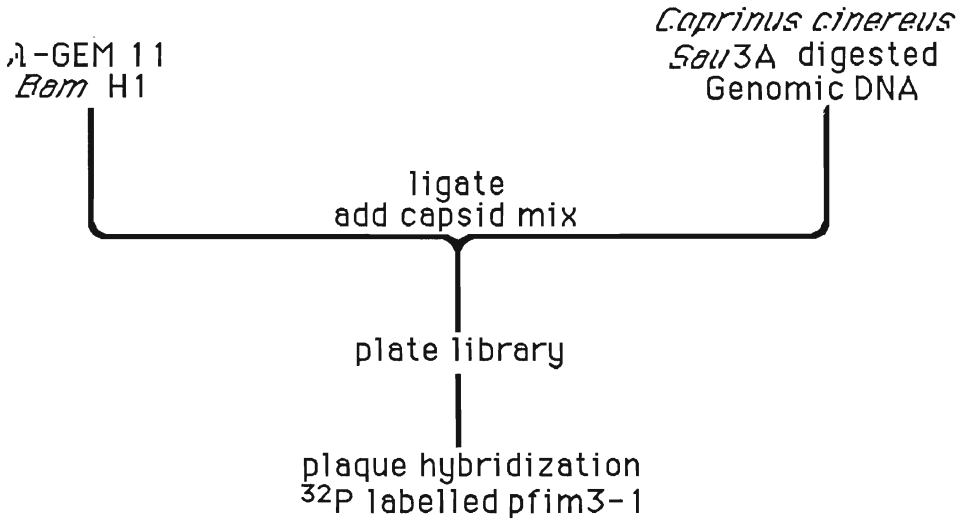
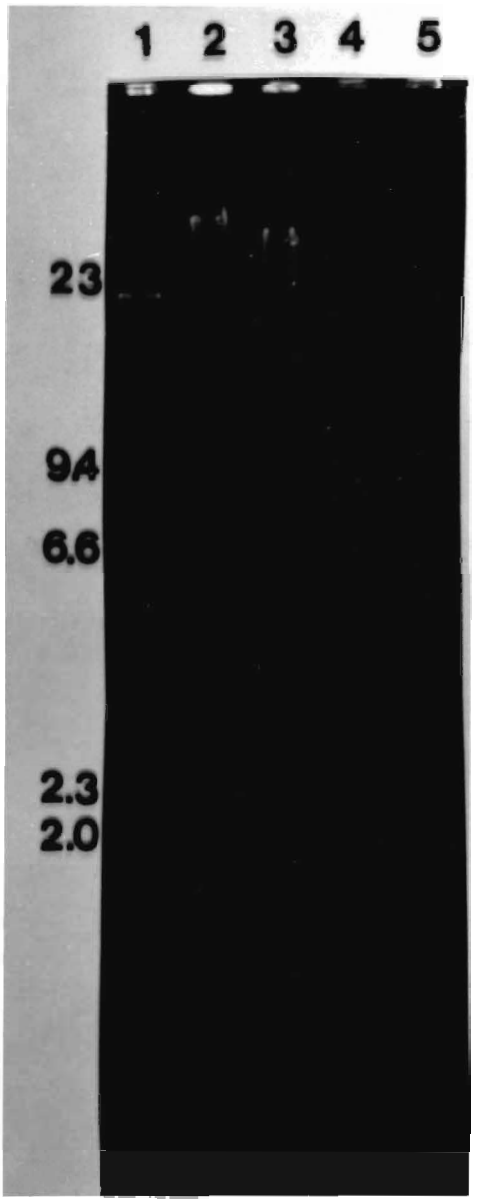


Figure 4. **Cloning strategy for isolation of DNA homolous to fimbrial cDNA.**

Coprinus cinereus DNA was digested with *Sau* 3A restriction endonuclease under conditions which generate a high proportion of fragments between 8 and 20 kbp. Fragments were ligated to arms of λGEM-11 vector utilizing the *Bam* HI restriction sites. The ligated DNA was packaged into capsid particles and grown lytically on a lawn of *E. coli* strain LE 392 cells. Plaque hybridization with ^{32}P labelled pfim3-1 was used to show homology between the cDNA and cloned DNA fragments from *C. cinereus*.

Figure 5. ***Sau*3A digestion of *C.cinereus* DNA.**

CsCl purified DNA from *C. cinereus* was digested with *Sau* 3A, separated by agarose gel electrophoresis and stained with ethidium bromide. Lane 1 contains *Hin* dIII digested λ as molecular weight markers. Lane 2 is undigested *C. cinereus* genomic DNA. Genomic DNA was digested with *Sau* 3A for 1 min (lane 3), 3 min (lane 4) and 5 min (lane 5). The 5 min digestion yielded size fragments between 5 and 23 kbp. This fraction was selected for library construction. The molecular masses of the standards in lane 1 are indicated on the left in kbp.


1
2
3
4
5
23
9.4
6.6
2.3
2.0

392 cells on each of 5 LB plates. Nylon membranes were laid over the plates to allow adhesion of lysed cells including bacteriophage DNA to the membrane. Plaques showing homology to both pfim3-1 and pfim9-1 (fimbrial cDNAs) were identified by hybridization of ^{32}P labelled cDNAs to the membranes. 22 plaques showed homology to one of the cDNAs; none showed strong homology to both.

The putative fimbrial plaques were removed, diluted in SM and successively purifed three times by reinfecting *E. coli* strain LE392 cells. At each stage these were screened by plaque hybridization to both cDNAs. A single clone, λ2-3A, showed strong hybridization of pfim3-1 to all plaques following the successive stages of hybridization (figure 6).

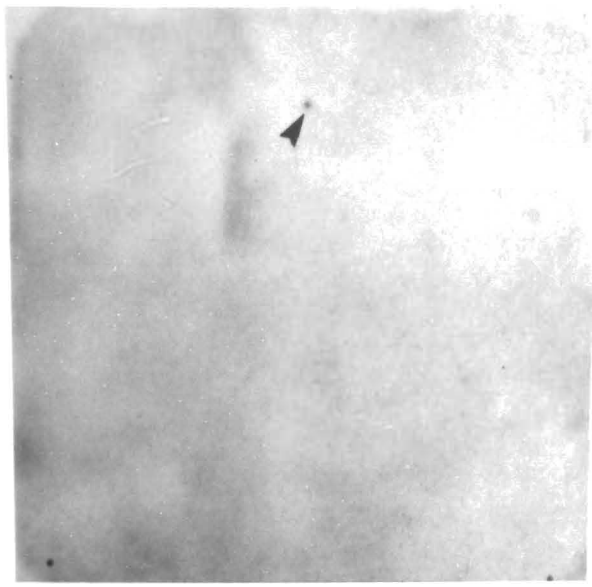
Localization of *C. cinereus* Sequence Showing Homology to cDNA

λ2-3A DNA was extracted from cultures of infected LE 392 cells. Restriction digests were performed with*Hin* dIII, *Bam* HI, *Eco* RI or *Sac*I restriction enzymes and the fragments were separated by agarose gel electrophoresis. DNA was Southern blotted to nylon membranes. Hybridization of pfim3-1 was performed in order to localize the DNA fragments with homology to the cDNA. pfim3-1 was found to be homologous to a single fragment from each digest (figure 7).

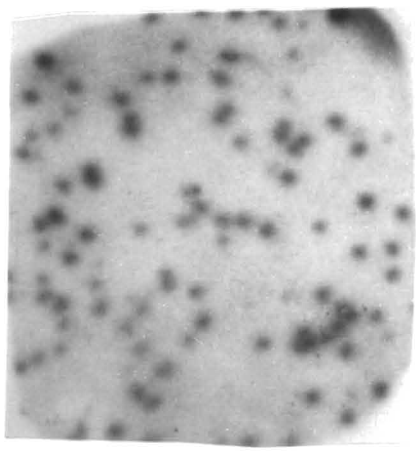
The *Sac* I fragment of λ2-3A DNA of fungal origin was cloned into pUC19. The region of homology to pfim3-1 was localized to a *Sac* I/ *Bam* HI fragment within pSS1 (figure 8). Successive stages of subcloning of fragments homologous to pfim3-1 was made ultimately yielding a 700 bp *Xba* I fragment. The plasmid containing this fragment was called pXX8 (figure 9).

Figure 6. **Library Screening for homology to pfim3-1.**

Approximately, 12500 cloned DNA fragments from a *C. cinereus* genomic library were plated on 5 LB-Mg plates with a lawn of *E. coli* strain LE392. Plaque hybridization with ^{32}P labelled pfim3-1 was performed (A). The arrow indicates a single plaque hybridizing strongly with pfim3-1. This clone was named λ2-3A. Plaques hybridizing with pfim3-1 were removed and replated. Those plaques which again showed hybridization were removed, plated dilutely (100 pfu/plate) with LE392 cells and similarly hybridized with the radiolabelled cDNA. All plaques from the plating of λ2-3A appeared to hybridize with pfim3-1 (B).



A



B

Figure 7. **Southern blots of restriction digested λ2-3A.**

λ2-3A was restriction endonuclease digested with *Hin* dIII (lane 1), *Bam* HI (lane 2), *Eco* RI (lane 3) and *Sac* I (lane 4). The DNA fragments were separated by agarose gel electrophoresis and transfered to nylon membranes. Radiolabelled pfim3-1 cDNA was hybridized to homologous DNA restriction fragments. The molecular masses are indicated in kbp to the left of the blots.

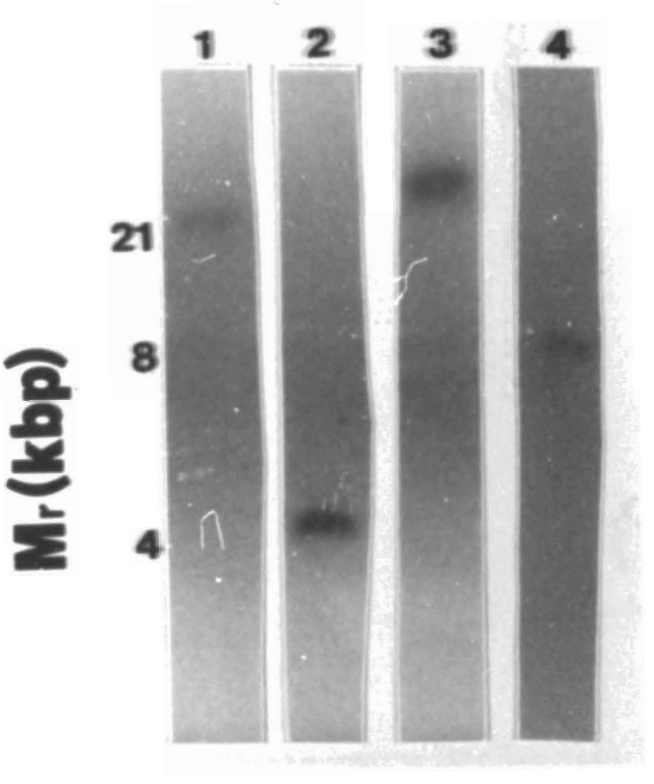

1
2
3
4
21
8
4
Mr(kbp)

Figure 8. **Localization of the sequences homologous to pfim3-1 within pSS1.**

pSS1 insert DNA was digested with *Hin* dIII (lane 1), *Hin* dIII and *Bam* HI (lane 2) and *Bam* HI (lane 3), separated by agarose gel electrophoresis, transfered to nylon membrane and hybridized with radiolabelled pfim3-1 (A). The restriction map of the pSS 1 fragment is shown (B) with the shaded *Sac* I-*Bam* HI fragment corresponding to the area of homology to pfim 3-1. Restriction endonuclease recognition sites are indicated by S (*Sac* I), B (*Bam* HI), X (*Xba* I) and H (*Hin* dIII). The molecular masses of hybridizing DNA fragments are indicated in kbp on the left.

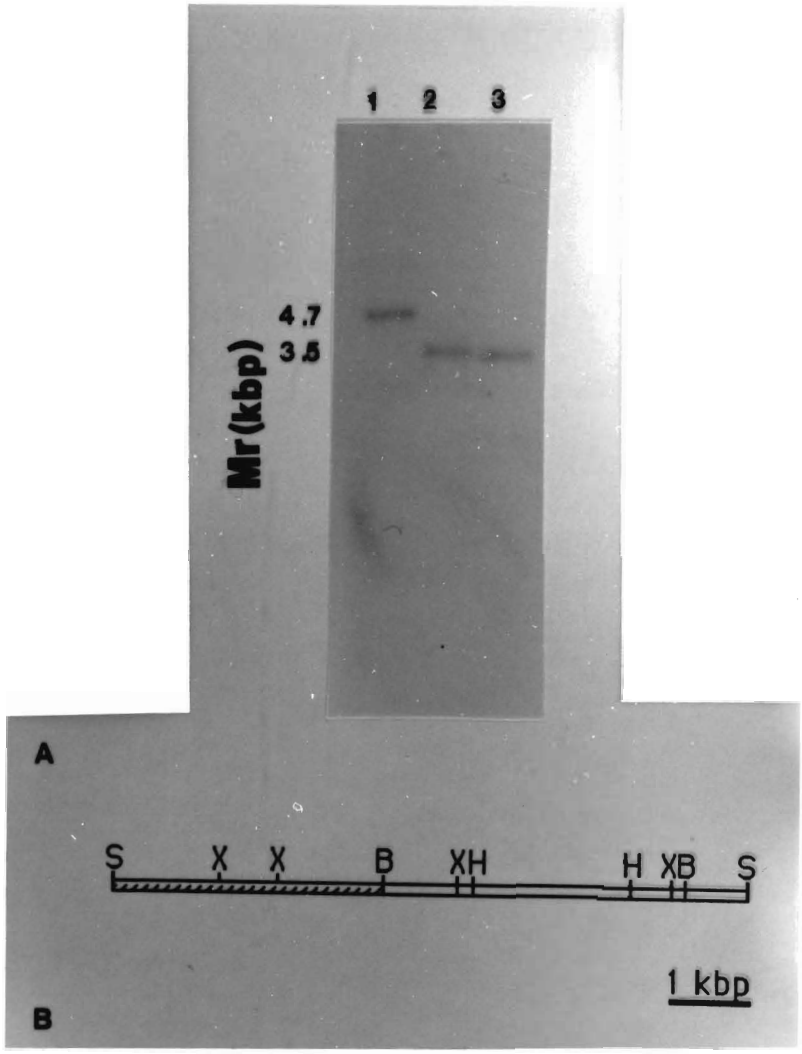
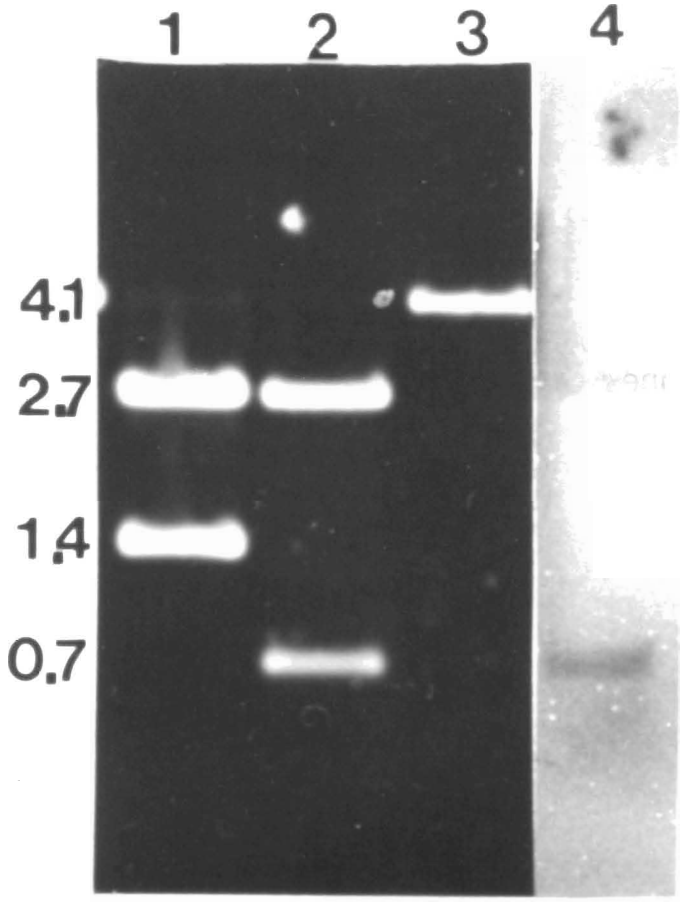

1 2 3
Mr(kbp)
4.7
3.5
A
S X X B XH H XB S
1 kbp
B

Figure 9. **Localization of the region of homology within a 700bp cloned DNA insert.**

The *Sac* I-*Bam* HI fragment which showed homology to pfim3-1 was subcloned into pXX9 (lane 1), pXX8 (lanes 2 and 4) and pSX 5 (lane 3). These were restriction digested with *Xba* I and separated by agarose gel electrophoresis. Lanes 1, 2 and 3 are stained with ethidium bromide. The 700bp pXX8 insert in lane 4 was transfered to a nylon membrane and hybridized to ^{32}P labelled pfim3-1. The pXX8 insert was found to hybridize to pfim3-1. The molecular masses of DNA fragments are indicated in kbp on the left.


1
2
3
4
4.1
2.7
1.4
0.7

Hybridization of cDNA and *C. cinereus* Sequences to RNA

RNA was extracted from both *C. cinereus* (strain 6323) and *S. commune* (strain 1378). RNAs were separated by formaldehyde agarose gel electrophoresis. The separated RNAs were transfered to nylon membranes, fixed and hybridized with radiolabelled pXX8 or pfim3-1. Both of these probes were found to hybridize to transcripts of the same size in both species, 1900+-60 nucleotides in length as determined by the relative migration of 18S and 26S ribosomal RNAs (figure 9).

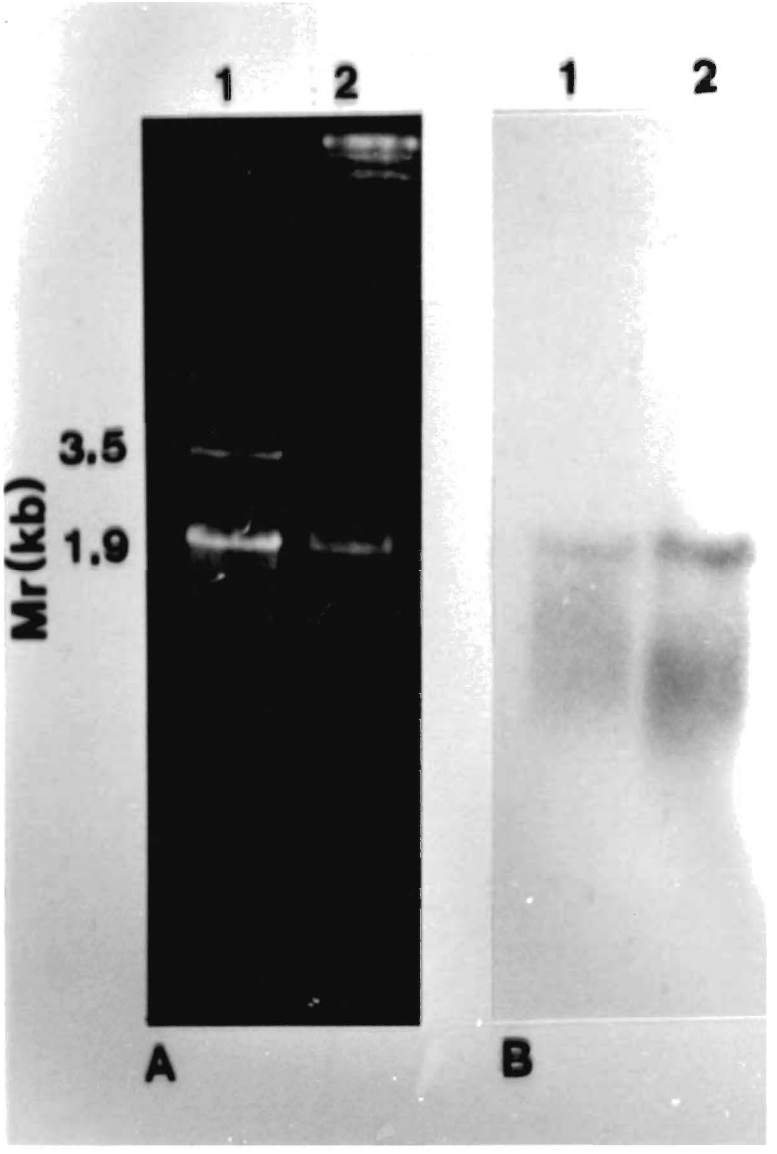
Sequence of *C. cinereus* DNA Showing Homology to Fimbrial cDNA

DNA sequence determinations were made by the dideoxy chain termination method of Sanger (1977). pXX 8 insert which was found to have homology to pfim 3-1 was sequenced. The plasmid clones derived from adjacent sequences in λ2-3A were also sequenced from their common restriction site towards distal sequences to a total length of 1167 bp (figure 11). The compiled sequence (figure 12) was then submitted to analysis by the FASTA nucleic acid analysis program (courtesy of European Molecular Biology Laboratory, Heidelberg). The nucleic acid sequences were compared to known sequences. The sequence was found to be approximately 97% similar to small subunit ribosomal DNA from *C. cinereus* .

The *C. cinereus* DNA sequence, that hybridized with pfim3-1, obtained from the genomic DNA library appears to be ribosomal DNA (rDNA). The reason for hybridization of pfim3-1 to rDNA, a completely unexpected result, was examined by determination of the nucleic acid sequence of the cDNA.

Figure 10. **Hybridization of pfim3-1 to RNA of *C. cinereus* and *S. commune*.**

RNA from *C. cinereus* (1) and *S. commune* (2) were separated by formaldehyde agarose gel electrophoresis, stained with ethidium bromide (A) and transfered to a nylon membrane (B). Transfered RNAs were hybridized with ^{32}P labelled pXX8. A single transcript of 1900±60 bases was identified in both *C. cinereus* and *S. commune*. The molecular masses of RNAs are indicated in kb on the left and were estimated from the relative migration of fragments to the rRNAs.


1
2
1
2
3.5
1.9
Mr(kb)
A
B

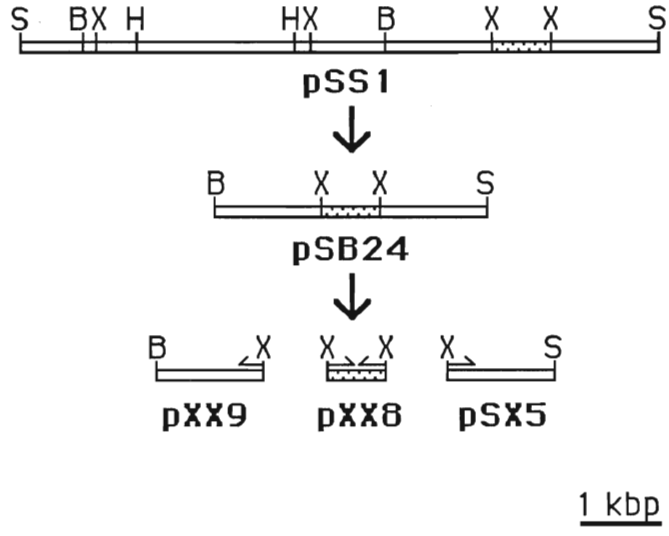


Figure 11. **Sequence strategy for DNA fragments isolated from *C. cinereus*.**

Restriction enzyme sites for *Bam* HI (B), *Hin* dIII (B), *Sac* I (S) and *Xho* I (X) of pSS1 insert and the subcloned pSB24, pXX9, pXX8 and pSX5 are shown. Subcloning stages are indicated by vertical arrows. The hatched domain represents the *Xho* I fragment which was found to hybridize to pfim3-1. The horizontal arrows adjacent to *Xho* I sites represents the direction that sequence information was obtained from each of these subclones.

Figure 12. **Nucleotide sequence of the cloned *C. cinereus* sequences showing homology to pfim3-1.**

The continuous sequence determined for pXX8, pXX9 and pSX5 are given. Comparison with sequence contained in the EMBL database indicates that the cloned DNA fragment from *C. cinereus* is a fragment of the rDNA repeat encoding the small subunit rRNA. The first base in the mature rRNA is designated by the number 1. The relative position of other bases are indicated in the right margin. The abbrieviations used to represent nucleotides are adenine (A), cytosine (C), guanine (G) and thymine (T).

CTGACGCTAG TACTCTCGAG AAGCATTCAG TTCAGTGATC TATCACCGAT GACGACTCCG -120

ACCGAGAAAC GCTTGCCTGC ACTATCGGGC GCGCTTCGGT GTGCAAGGAC GGAAAGTAGT -60

1

TACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT 60

AAGTATAAAC AACTTTGTAC TGTGAAACTA GCAATGGCTC ATTAAATCAG TATAGTTGTA 120

TTTGATGGTA TCTTACTACA TGGATAACAT GATCGGTAAT TCTAGAGCTA ATACATGCAA 180

TCAAGCCCCG ACTTCCGGAA GGGTGTATTT ATTAGATAAA AAACCAACGC GGCTCGCTGC 240

CCTTGGTGAT TCATAACTTC TCGAATCGCA TCGGCCTTGT CGGGCGATGC TTCATTCAAA 300

TATCTGCCCT ATCAACTTTC GATGGTAGGA TAGTGGCCTA CCATGGTTTC AACGGGTAAC 360

GGGGAATAAG GGTTCGATTC CGGAGAGGGA GCCTGAGAAA CGGCTACCAC ATCCAAGGAA 420

GGCAGCAGGC GCGCAAATTA CCCAATCCCG ACACGGAGGT AGTGACAATA AATAACAATA 480

TAGGGCTCTT TTGGGTCTTA TAATTGGAAT GAGTACAATT TAAATCCCTT AACGAGGAAC 540

AATTGGAGGG CAAGTCTGGT GCCACAGCCG CGGTAATTCC AGCTCCAATA GCGTATATTA 600

AAGTTGTTGC AGTTAAAAAG CTCGTAGTTG AACTTCAGAC CTGGCTGGGC GGCGAACGGC 660

GTGTACTCTC TGCTGGGCCT TACCTCTTGG TGAGCCGGCG TGCCCTTTAT TGGTGTGCGT 720

CGGGGAACCA GGACTTTTAC CTTGAGAAAA TTAGAGTGTT CAAAGCAGCC TTTGCCCGAA 780

TACATTAGCA TGGAATAATA AAATAGGACG TGCGGTTCTA TTTTGTTGGT TTCTAGAGTC 840

GCCGTAATGA TTAATAGGGA TAGTTGGGGG CATTGGTATT GAGTCGCTAG AGGTGAAATT 900

CTTGGATTGA CTCAAGACCA ACTACTGCGA AACGATTTCC AAGGATGTTT TCATTAATCA 960

AGAACGAAGG TTAGGGGATC GAAAACGATT AGATACCGTT GTAGTCTAAC AGTAAACTAT 1020

GCCGACTAGG ATCGAGACGA GTATTTA 1047

Sequence of Fimbrial cDNA of *U. violacea*

The pfim3-1 fimbrial cDNA was subcloned into a variety of different sized fragments. *Eco* RI and *Sac* I were used simultaneously to cut pfim3-1 into 2 fragments (700 and 800 bp). *Eco* RI and *Bam* HI were similarly used to generate fragments of 1000 and 500 bp in length (figure 13). The four fragments generated were subcloned into pUC19 and named pfim3-1ES2, pfim3-1ES1, pfim3-13 and pfim3-15. DNA sequence determinations were performed on all four pfim3-1 derived clones to generate a complete DNA sequence for pfim3-1 (figure 14).

Analysis of the nucleic acid sequence yielded a 576 bp domain (nucleotides 780 to 1355) with 81% homology to pXX8 (nucleotides 319 to 879) (figure 15) and 83% homology to the published sequence of *U. maydis* 18S ribosomal genes (nucleotides 315 to 878). As determined using using the melting temperature equation ($Tm= 81.5+16.6 \log[Na^+] + 0.41(\%G+C) + 500/length - \%base\ mismatch$) (Bonner et al., 1973), the hybridization conditions allow for a 28% base mismatch. Thus, the extent of homology between pfim3-1 and pXX8 is sufficient to account for the hybridization observed.

The sequence contained an approximately 100 base pair region of adenine-thymine base pairs. This region likely corresponds to a polyadenylated tail (poly-A tail) of mRNA. This region was used as an indicator of the DNA strand coding the fimbrial protein. A tripartite polyadenylation consensus sequence, *TAG...TATGT...TTT*, is found in genes from *Saccharomyces cerevisiae* (Zaret and Sherman, 1982) and the 2μm plasmid of *S. cerevisiae* (Sutton and Broach, 1985). Similar sequences are found in the 3' untranslated regions of genes from

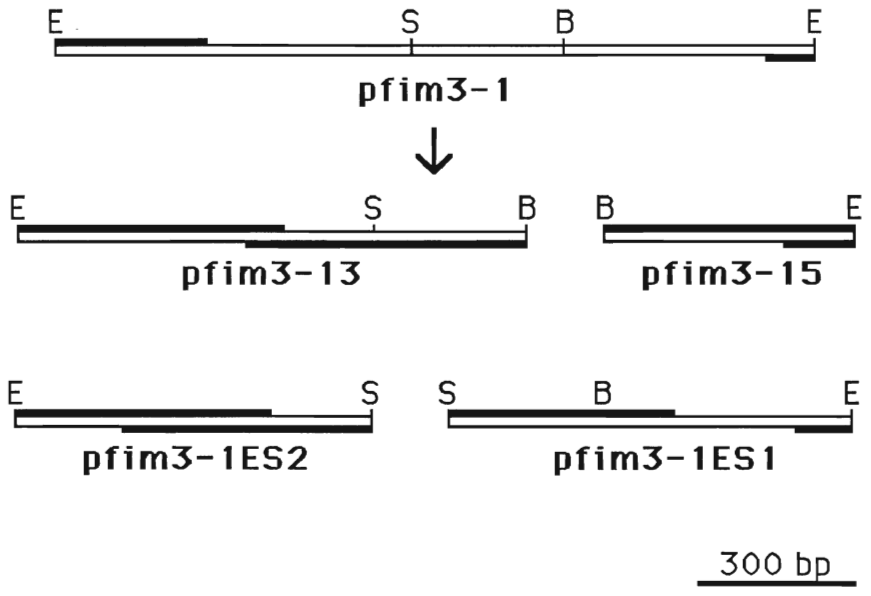


Figure 13. **DNA sequence strategy for pfim3-1.**

Restriction enzyme maps of pfim3-1 and subclones (pfim3-13, pfim3-15, pfim3-1ES1 and pfim3-1ES2) are shown. These clones were used to generate a complete sequence of pfim3-1. The open bars represent the double stranded DNA insert cloned into pUC19. The solid bars indicate the sequence information obtained from each clone using primers which are complementary to sequences from pUC19. B, E, and S represent *Bam* HI, *Eco*RI and *Sac*I restriction endonuclease recognition sites.

Figure 14. **DNA sequence of pfim3-1 insert.**

The 1457 nucleotide DNA sequence determined for pfim3-1 is shown. The nucleotides are number (in the right margin) from 1 through 1457. Base 1 is the 5' terminal base on the DNA strand containing the putative 3' polyadenylation sequence (nt 1368 through 1457). The abbreviations used to represent nucleotides are adenine (A), cytosine (C), guanine (G) and thymine (T).

TTCTCGTGTGTCTTATTTCATTTCCTCCAGTGTTCGTGTCTCCTTCAAGAGGATCGCCTG 60

ACCTGTTTTGATAAGCACTCATCGCGATTTCAAAAAAAAGGCCGCTCTTCCTCTTCACTT 120

CTGTTATTTTCCTTGACCACGCGCGACGCTTTACTTGTTCTTCTCGGCGTGCTCGGCGAA 180

CGTCTTTTGGAGACGTCTCCACACGGTCTTGGGGGACTGGGCCGCATCGACACCCTTCCA 240

GATGCCTTTCGGTCTTGTAGTAACTGCGACGGGGTCGGTCTGCTGGTGGTAGCGCTCGAG 300

GCGCTTCGAGAGGTCTCGGCCGCGTCGTCTGGAGCGTTGGATCAGGGTTCGCCCGTATGT 360

CGTCGGTCATGGCCTTTTTCGGGGGCAAACTCCTTGTGGTACGAACGACCGGAAGCGGGG 420

TGGATCAAACGACCGGTGATGCGGCTGACGAGCAAGTTGTCGTTGACAAGCAACTGGATC 480

GCGTGCTCGAGGGGTTGTTGTCCTCCCTCGAGCATGCTGTCGAGCTTCTGGGCCTGGCCG 540

ACCGTGCGAGGGAAACCGTCGAGGATGAAACCAAGCTGCACTCCTTATTCTCGTCCAACT 600

GCTGCTGGATCATGCCGATGACGATCTCATCCCGAGACGAGGCCACCTTGGTCCATGATC 660

TTCTTAGCCTGCTTGCGAGCTCGAGCTCGGTGCCTTGCTTGACCTGCTCGCGTAACATGT 720

CGCCGGTCGCCAAGTGGCAGACGTTGAACTTGTCCTTGATGTTGGGCGCTTGGGTCCCTT 780

CGATGGTAGGATAGAGGCCTACCATGGTGATGACGGGTAATGGGGAATAAGGGTTCGATT 840

CCGGAGAGAGGGCCTGAGAAACGGCCCTCAGGTCTAAGGACACGCAGCAGGCGCGCAAAT 900

TATCCCCTGGCAACACTTTGCCGGAGATAGTGACAATAAATAACAATGCAGGGCTCTTAC 960

GGGTCTTGCAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGATCCATTGGAGGGCA 1020

AGTCTGGTGCCAGCAGCCGCGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCCG 1080

TTAAAAAGCTCGTAGTCGAACTTCGGTCCCTGTAAGCCGGTCCGCCTTCTTGGTGTGTAC 1140

TTGCCTCTGCGGGGACTTACCTCCTGGTGAACTGCCATGTCCTTTACTGGGTGTGGTAGC 1200

AAACCAGGACGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCCTACGCCCGAATAC 1260

ATTAGCATGGAATAATAGAATAGGACGCGCCGTTCCCATTTTGTTGGTTTCTCGAGATCG 1320

CCCGTAATGATTAATAGGAACTGGGGGCAATATGATGAACGTTCTACAAAAAAAAAAAAA 1380

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1440

AAAAAAAAAAAAAAAA 1457

Figure 15. **pfim3-1 nucleic acid sequence homologous to *C. cinereus* rDNA.**

The DNA sequences of pfim3-1 and pXX8 are aligned. The upper sequence is that determined for pXX8. Base substitutions in pfim3-1 relative to pXX8 are indicated by the lower sequence. Conserved bases are indicated by spaces and bases not represented within the sequence are indicated by '-'. The extent of nucleic acid homology between pfim3-1 and pXX8 was determined to be 81%. Alignments were performed manually to generate maximum similarity where similarity was estimated as [1-(#mismatch/# of nt in published sequence)]x100%. The abbreviations used to represent nucleotides are adenine (A), cytosine (C), guanine (G) and thymine (T).

```
TTCGATGGTA  GGATAGTGGC  CTACCATGGT  TTCAACGGGT  AACGGGGAAT  AAGGGTTCGA
                  A                 GATG          T

TTCCGGAGAG  GGAGCCTGAG  AAACGGCTAC  CACATCCAAG  GA AGGCAGC  AGGCGCGCAA
            A G                 CT    GG  T     C C

ATTACCCAAT  CCCGA-----  CACGGAGGTA  GTGACAATAA  ATAACAATAT  AGGGCTCTTT
       CTG  G AACACTTT  C      A                       GC           A

TGGGTCTTAT  AATTGGAATG  AGTACAATTT  AAATCCCTTA  ACGAGGAACA  ATTGGAGGGC
C       GC                                              T  C

AAGTCTGGTG  CCACAGCCGC  GGTAATTCCA  GCTCCAATAG  CGTATATTAA  AGTTGTTGCA
                                                           C

GTTAAAAAGC  TCGTAGTTGA  ACTTCAGACC  TGGCTGGGCG  GC--GA--    ACGGCGTGCT
                  C           G T     TAA C-    TCC CCTTC  TT  T   TA

TGTTACTCTC  TGCTGGGC-C  TTACCTCTTG  GTGAGCCGGC  GTGCCCTTTA  TT GGTGTGC
-C  G -        -    GA          C       A T C  C  T        C-G      -

GTCGGGGAAC  CAGGACTTTT  ACCTTGAGAA  AATATAGAGT  GTTCAAAGCA  GCCTTTGCCC
  A CA-           G       T    A      -G                        AC

GAATACATTA  GCATGGAATA  ATAAAATAGG  ACGTGCGGTT  CT ATTTTGT  TGGTTTCT A
                           G          C  C      CC                 C

GAGTCGCC G  TAATGATTAA  TAGGGATAGT  TGGGGGCATT  GGTAT
  -     C                   A----C          A   ATG
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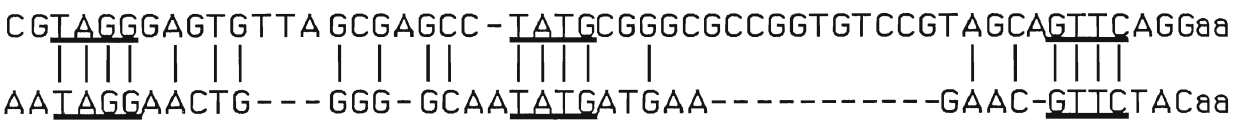

Figure 16. **Putative polyadenylation signals in fimbrial cDNAs.**

The DNA sequences of the 3' ends of pfim9-1 (upper) and pfim3-1 (lower) are presented. Lower case 'a' s denote the position of the polyadenylation sequence. The underlined sequences indicate a tripartite polyadenylation signal commonly found in fungal genes (Zaret and Sherman, 1982). Vertical lines represent nucleotide identities. The abbreviations used to represent nucleotides are A (adenine), C (cytosine), G (guanine) and T (thymine).

Aspergillus nidulans (Bradshaw and Pillar, 1991) and *U. maydis* (Tao et al., 1990; Keon et al., 1991). A similar sequence is present adjacent to the polyadenylation sequence of pfim3-1 between nucleotides 1335 and 1361, *TAGG...TATG...GTTC* (figure 16). The presence of this sequence adjacent to a region of adenine residues in both pfim9-1 (Castle, personal communication) and pfim3-1 suggests that this may be the signal required for polyadenylation of mRNAs in *U. violacea*.

Deduced Amino Acid Sequence of Fimbrial Protein

Cloned cDNAs in λgt-11 are expressed as β-galactosidase fusion proteins (Young et al., 1983). A deduced amino acid sequence in the reading frame of the fusion protein was generated from the DNA sequence of pfim3-1. The translation product was deduced in this reading frame as no other openreading (ORF) was present within the sequence. A 206 amino acid polypeptide with a predicted molecular mass of 22 kDa is encoded by the cDNA fragment (figure 17). This represents approximately 1/3 of the molecular mass of the fimbrial protein without glycosylation (Castle et al., 1992). The amino acid sequence was submitted to FASTA protein analysis. Two amino acid domains were found to be similar to sequences contained in the database.

A 33 amino acid domain, amino acids 42 through 74, from the translated pfim3-1 nucleic acid sequence was found to be similar to a hydrophobic domain in rat leukocyte surface antigen CD53. There is 30% identity in amino acid sequence and a further 39% similarity in terms of conservative amino acid substitutions (figure 18). This

Figure 17. **Deduced amino acid sequence from pfim3-1.**

The deduced amino acid sequence for pfim3-1 was derived from the DNA sequence determined. The amino acids are listed above the corresponding codon. The amino acid and nucleotide positions are listed in the right margin. The truncated open reading frame encodes a 207 amino acid polypeptide. The position of the amino acids are indicated in the right margin. The abbreviations used to represent amino acids are A (alanine), C (cysteine), D (aspartic acid), E (glutamic acid), F (phenylalanine), G (glycine), H (histidine), I (isoleucine), K (lysine), L (leucine), M (methionine), N (asparagine), P (proline), Q (glutamine), R (arginine), S (serine), T (threonine), V (valine), W (tryptophan), Y (tyrosine) and Z (stop codon). The abbrieviations used to represent nucleotides are adenine (A), cytosine (C), guanine (G) and thymine (T).

```
F  S  C  V  L  F  H  F  L  Q  C  S  C  L  L  Q  E  D  R  L    20
TTCTCGTGTGTCTTATTTCATTTCCTCCAGTGTTCGTGTCTCCTTCAAGAGGATCGCCTG  60
T  C  F  D  K  H  S  S  R  F  Q  K  K  G  R  S  S  S  S  L    40
ACCTGTTTTGATAAGCACTCATCGCGATTTCAAAAAAAAGGCCGCTCTTCCTCTTCACTT 120
L  L  F  S  L  T  T  R  D  A  L  L  V  L  L  G  V  L  G  E    60
CTGTTATTTTCCTTGACCACGCGCGACGCTTTACTTGTTCTTCTCGGCGTGCTCGGCGAA 180
R  L  L  E  T  S  P  H  G  L  G  G  L  G  R  I  D  T  L  P    80
CGTCTTTTGGAGACGTCTCCACACGGTCTTGGGGGACTGGGCCGCATCGACACCCTTCCA 240
D  A  F  R  S  C  S  N  C  D  G  V  G  L  L  V  V  A  L  E   100
GATGCCTTTCGGTCTTGTAGTAACTGCGACGGGGTCGGTCTGCTGGTGGTAGCGCTCGAG 300
A  L  R  E  V  S  A  A  S  S  G  A  L  D  Q  G  S  P  V  C   120
GCGCTTCGAGAGGTCTCGGCCGCGTCGTCTGGAGCGTTGGATCAGGGTTCGCCCGTATGT 360
R  R  S  W  P  F  S  G  A  N  S  L  W  Y  E  R  P  E  A  G   140
CGTCGGTCATGGCCTTTTTCGGGGGCAAACTCCTTGTGGTACGAACGACCGGAAGCGGGG 420
W  I  K  R  P  V  M  R  L  T  S  K  L  S  L  T  S  N  W  I   160
TGGATCAAACGACCGGTGATGCGGCTGACGAGCAAGTTGTCGTTGACAAGCAACTGGATC 480
A  C  S  R  G  C  C  P  P  S  S  M  L  S  S  F  W  A  W  P   180
GCGTGCTCGAGGGGTTGTTGTCCTCCCTCGAGCATGCTGTCGAGCTTCTGGGCCTGGCCG 540
T  V  R  G  K  P  S  R  M  K  P  S  C  T  P  Y  S  R  P  T   200
ACCGTGCGAGGGAAACCGTCGAGGATGAAACCAAGCTGCACTCCTTATTCTCGTCCAACT 600
A  A  G  S  C  R  Z                                          207
GCTGCTGGATCATGCCGATGACGATCTCATCCCGAGACGAGGCCACCTTGGTCCATGATC 660

TTCTTAGCCTGCTTGCGAGCTCGAGCTCGGTGCCTTGCTTGACCTGCTCGCGTAACATGT 720

CGCCGGTCGCCAAGTGGCAGACGTTGAACTTGTCCTTGATGTTGGGCGCTTGGGTCCCTT 780

CGATGGTAGGATAGAGGCCTACCATGGTGATGACGGGTAATGGGGAATAAGGGTTCGATT 840

CCGGAGAGAGGGCCTGAGAAACGGCCCTCAGGTCTAAGGACACGCAGCAGGCGCGCAAAT 900

TATCCCCTGGCAACACTTTGCCGGAGATAGTGACAATAAATAACAATGCAGGGCTCTTAC 960

GGGTCTTGCAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGATCCATTGGAGGGCA 1020

AGTCTGGTGCCAGCAGCCGCGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCCG 1080

TTAAAAAGCTCGTAGTCGAACTTCGGTCCCTGTAAGCCGGTCCGCCTTCTTGGTGTGTAC 1140

TTGCCTCTGCGGGGACTTACCTCCTGGTGAACTGCCATGTCCTTTACTGGGTGTGGTAGC 1200

AAACCAGGACGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCCTACGCCCGAATAC 1260

ATTAGCATGGAATAATAGAATAGGACGCGCCGTTCCCATTTTGTTGGTTTCTCGAGATCG 1320

CCCGTAATGATTAATAGGAACTGGGGGCAATATGATGAACGTTCTACAAAAAAAAAAAAA 1380

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1440

AAAAAAAAAAAAAAAA                                              1457
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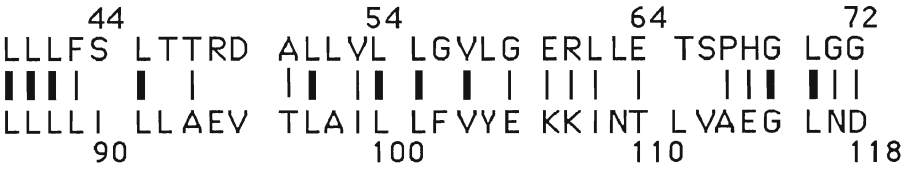

Figure 18. **Comparison of deduced amino acid sequence to the amino acid sequence of rat CD53.**

The deduced amino acid sequence for pfim3-1 was compared to sequences compiled in the EMBL database using the FASTA computer program. The 33 amino acid domain above shows 30% identity and 69% similarity in terms of conservative base substitutions. Thick vertical lines represent amino acid identities between the deduced sequence (upper) and the sequence of rat leukocyte antigen CD53 (lower). Conservative base substitutions are represented by thin vertical lines. The amino acid abbreviations are: A (alanine), D (aspartic acid), E (glutamic acid), F (phenylalanine), G (glycine), H (histidine), I (isoleucine), K (lysine), L (leucine), M (methionine), N (asparagine), P (proline), R (arginine), S (serine), T (threonine, V (valine) and Y (tyrosine).

domain in CD53 is a membrane spanning domain (Horejsi and Vlcek, 1991).

A 20 amino acid hydrophilic domain, 130 through 149, was found to be similar to domains in DNA polymerase of Herpes Simplex Viruses (Gibbs et al., 1985), NS4a protein from the Dengue viruses (Mackow et al., 1987; Hahn et al., 1988) and human thrombospondin (Lawler et al., 1991). These domains have not been characterized sufficiently to draw analogies to the corresponding domain in the fimbrial protein of *U. violacea*. Thus, the function of this domain remains obscure.

The DNA sequence information generated for pfim3-1 and pXX8 are summarized in figure 19. pfim3-1 was found to contain a terminal region of adenine-thymine base pairs. This region was assumed to represent a polyadenylation sequence and was thus used to assess the 5' to 3' orientation of the expected fimbrial transcript. An incomplete ORF was found at the 5' end of pfim3-1. This incomplete ORF was separated from the polyadenylation sequence by a 576 nucleotide untranslated domain which showed homology to pXX8. pXX8 and the homologous domain in pfim3-1 both show homology to rDNA encoding the small subunit rRNA.

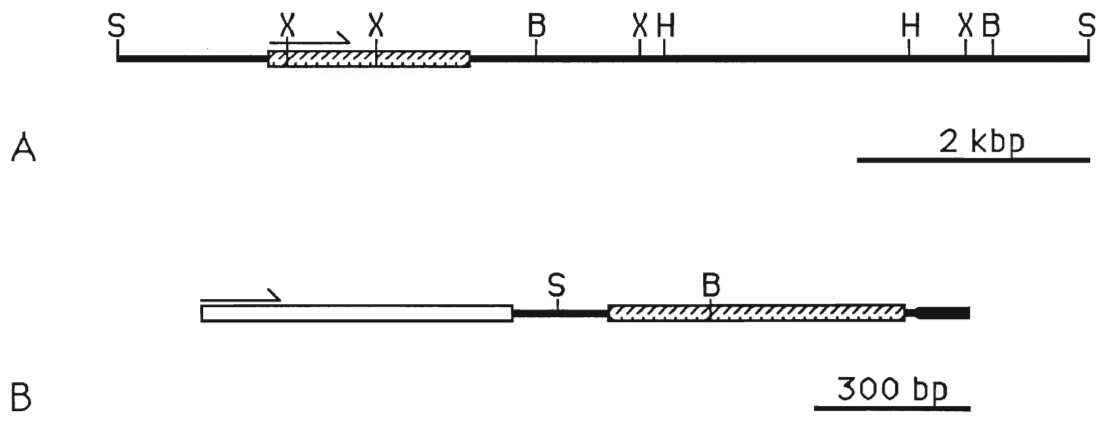

Figure 19. **Summary of DNA sequence information.**

DNA sequence information derived from pSS1 insert (A) and pfim3-1 insert (B) are summarized. The small subunit ribosomal sequence cloned into pSS1 is indicated by the cross hatched domain. Domains within pfim3-1 are differentiated by an open box (partial ORF), a cross hatched box (domain homologous to rDNA) and a black box (polyadenylation sequence). The 5' to 3' direction of transcripts is indicated by the arrows. The position of restriction endonuclease recognition sites is indicated for *Sac* I (S), *Bam* HI (B), *Hin*dIII (H) and *Xba* I (X).

Discussion

Coprinus cinereus and *Schizophyllum commune* are genetically the best characterized basidiomycetes. Transformation systems have been developed for these fungi (Binninger et al., 1987; Munoz-Rivas et al, 1986), thus yielding a tool for the initiation of studies of fimbrial gene regulation in filamentous basidiomycetes. The confirmation of fimbriae on hyphae of both *C. cinereus* and *S. commune* and the identification of fimbrial proteins of *C. cinereus* provide a basis for the initiation of such studies (Boulianne, 1990).

Prior to nucleic acid analysis of the fimbrial gene of *C. cinereus*, the fimbrial antigen had to be confirmed in the strains selected in order to ensure that the presence of fimbriae is not a polymorphic character within this species. Immunoblot analysis of total soluble proteins (TSP) from *C. cinereus* revealed 37 and 39 kDa proteins recognized by the polyclonal antiserum raised against the fimbrial proteins of *Ustilago violacea*. The 37 kDa protein had been identified as the fimbrial subunit (Boulianne, 1990). The confirmation of fimbrial antigen was interpreted as expression of fimbriae. Thus, fimbriae have been observed on all isolates of *C. cinereus* examined to date.

Previous attempts to identify the fimbrial proteins of *S. commune* were unsuccessful. Boulianne (1990) observed only inconsistent and very weak binding of AU to large molecular weight peptides from strains that have been commonly used in genetic studies. A 50 kDa polypeptide from recently collected wild isolates of *S. commune* was identified. The characteristics of this antigenic peptide were examined in order to compare this protein to the fimbrial proteins from other species. Two dimensional gel electrophoresis of TSP

revealed three isoforms with isoelectric points ranging between 5.6 and 5.8. The existence of multiple isoforms had previously been shown for *U. violacea* (Castle et al., 1992) and *C. cinereus* (Boulianne, 1990). Isoforms found to be antigenic were found to be similar in isoelectric range to the fimbrial proteins of *U. violacea* (Castle et al, 1992) but dissimilar to those of *C. cinereus* (Boulianne, 1990). Thus, the charge of the fimbrial subunit does not appear to be conserved between species.

The lack of success in earlier attempts to identify the fimbrial proteins of *S. commune* (Boulianne, 1990) may reflect the age of the cultures used. The strain (8-3) used was not a recent wild type isolate but rather an auxotrophic laboratory strain maintained as a monokaryon (Boulianne, 1990). It could be that the role of fimbriae in unmated cultures is dispensable. The selection pressure for maintenance of fimbriae may be stage dependant in the life cycle of *S. commune.* Fimbriae are differentially expressed in a stage dependant manner in both *C. cinereus* and *U. violacea.* Oidia of *C. cinereus* and sporidia of *U. violacea* are more fimbriate than hyphae (Boulianne, 1990; Gardiner, 1985).

The fimbrial cDNAs from *U. violacea* were found to hybridize to genomic DNA of both *C. cinereus* and *S. commune.* This heterologous binding was used to identify a DNA sequence homologous to the *U. violacea* fimbrial cDNA from a *C. cinereus* genomic DNA library by plaque hybridization. FASTA computer analysis of the nucleic acid sequence determined for this clone was found to correspond to a fragment of the ribosomal repeat sequence coding the 18S rRNA.

The pfim3-1 cDNA was sequenced in order to determine if

sequences with homology to ribosomal DNA were present. Such a sequence would account for the hybridization of the fimbrial cDNA to the *C. cinereus* rDNA. A 576 bp domain within pfim3-1 insert showed 83% homology to the small subunit rDNA of *U. maydis* (De Wachter et al., 1992) and 81% homology to pXX8 insert. A domain of this size with the extent of homology present could account for the hybridization of pfim3-1 to ribosomal sequences of *C. cinereus* (Bonner et al., 1973).

The hybridization of pfim3-1 to rDNA favours the isolation of nonfimbrial DNA sequences as there are between 60 and 90 copies of the ribosomal repeat of *C. cinereus* per haploid genome (Cassidy et al., 1984). Hence, the strategy employed in the isolation of fimbrial genes through heterologous binding of pfim3-1 was inadequate due to the unanticipated homology to rDNA.

The remainder of pfim3-1 lacked significant homology to any known nucleic acid sequences. Preliminary reanalysis of clones derived from the *C. cinereus* DNA library indicated that several clones that hybridized to pfim3-1 hybridize to the *Eco*RI-*Sac*I DNA fragment from pfim3-1 that does not show homology to the small subunit rDNA (De Wachter et al., 1992). This sequence could still be used in order to attempt the isolation of the fimbrial genes from *C. cinereus* and *S. commune*.

The region of homology to the rRNA of *U. maydis* could be either an artifact of the cloning of the cDNA or a domain which is characteristic of the fimbrial gene of *U. violacea*. Such an artifact could have arisen in the cloning process in a number of ways. Firstly, reverse transcriptase required in the isolation of cDNAs may have copied portions of two RNA molecules that were associated at the time

of RNA isolation. Also, Blunt end ligation of linkers to the reverse transcription products could induce ligations of different cDNAs. Thirdly, a cross over could have occured such that the template copied was a chimera of mRNA and rRNA. However, several characteristics of the pfim3-1 DNA sequence suggest that it is unlikely that this region is the product of an artifact of the cloning process.

The region of homology is located between the incomplete open reading frame and a series of adenine residues which are presumed to represent the polyadenylation sequence characteristic of mRNAs. An error in reverse transcription yielding a domain such as this would require two separate reading errors. The reverse transcriptase would have to copy the 3' poly-A sequence followed by the rRNA sequence and the remainder of the mRNA.

Secondly, a consensus sequence required for the post transcriptional addition of adenine residues was found to be contained within the DNA sequence showing homology to 18S rRNA. The rRNAs of *U. maydis* and *C. cinereus* do not contain these nucleotide sequences.

Thirdly, *U. maydis* is the fungus most closely related to *U. violacea* (both are heterobasidiomycetes) for which the nucleotide sequence of the 18S rRNA is known. The nucleic acid homology between the 576 bp domain in pfim3-1 and the same domain in the 18S rRNA of *U. maydis* is 83%. This homology is less than that found between *U. maydis* (a heterobasidiomycete) and both *C. cinereus* (a homobasidiomycete) (87%) and *S. cerevisiae* (an ascomycete) (86%) (Rubstov et al., 1980). If the domain were derived from the reverse transcription of the 18S rRNA of *U. violacea*, one would expect that the nucleic acid sequences of *U. maydis* rRNA and pfim3-1 would show

stronger homology.

This evidence suggests that this region of similarity is native to the fimbrial cDNA, however, this will have to be shown conclusively. One should be able to distinguish these hypotheses using the hybridization of pfim3-1ES2 (the 5' end of pfim3-1) and pXX8 (the rDNA fragment from *Coprinus cinereus)* to both a common restriction fragment and transcript. This would indicate that these sequences are contiguous and transcribed as a unit.

The presence of large sequences homologous to rRNA is novel in eukaryotes, however, less extensive homologies between mRNAs and rRNAs have been described for prokaryotic genes. In *E. coli*, ribosomal protein (r-protein) mRNAs (rp-mRNAs) show nucleic acid homology to the 16S rRNA. The r-protein, S8, is involved in 30S ribosomal subunit assembly (Held et al., 1974) and is known to bind a specific domain within the 16S rRNA (Gregory et al., 1984). A 48 nucleotide region of the *spc* mRNA shows 20% identity in nucleic acid sequence but extensive secondary structural similarity to the S8 binding domain in the 16S rRNA (Gregory et al., 1988). S8 regulates its own expression through the binding of the protein to the transcript of the *spc* operon which encodes S8 and other r-proteins (Dean et al., 1981). Similar feedback inhibition mechanisms operate at the level of translation for *E. coli* r-proteins, S4 and S7 (29% homology to a 76 nt domain and 31% homology to a 135 nt domain, respectively) (Nomura et al., 1980).

The pfim3-1 sequence showing homology to the 16S-like rRNA is substantially larger and shows greater homology to the ribosomal sequence than the r-protein domains (Gregory et al., 1988; Nomura et al., 1980). The possibility exists that the fimbrial mRNA of *U. violacea*

has similar secondary structures to 16S-like rRNA. The ribosomal binding domains of r-proteins from fungi have not yet been determined to the same extent as in bacteria. The size of the homologous sequence and the presence of 32 r-proteins associated with the 40S ribosomal subunit (Bollen et al., 1981) makes it likely that the binding domains of a number of r-proteins are present in the fimbrial mRNA.

Ribosomal proteins may bind the fimbrial transcript to modulate expression of fimbrial protein. Production of fimbrial proteins must be sufficient to account for the rate of fimbrial extension following removal of fimbriae, 1-2μm/h/fibre (Gardiner and Day,. 1985), as *U. violacea* requires transciption for fimbrial synthesis (Poon and Day, 1974). The binding of r-proteins to the fimbrial mRNAs may aid in the efficient transport of fimbrial transcripts to ribosomes for rapid assembly of the initiation complex.

Following translation of rp-mRNAs in the cytoplasm, r-proteins are targeted for ribosome assembly in the nucleus (reviewed in Raué and Planta, 1991). Target sequences, nuclear localization signals (NLS), are present in both r-proteins and nuclear proteins. In *S. cerevisiae*, NLS defective proteins such as r-protein S24 and histone H2B, can be shuttled into the nucleus in association with a functional NLS containing protein (reviewed in Raué and Planta, 1991). Fimbrial transcript may bind targeted r-proteins in the nucleus such that fimbrial mRNA is in association with the 40S ribosomal subunit for rapid translational initiation. This may represent a completely novel mechanism of gene regulation in eukaryotes.

The initial *U. violacea* cDNA was cloned into a λgt11 expression vector (Castle, personal communication). As cloned sequences are

expressed as a β-galactosidase fusion protein, the correct reading frame for translation was known (Young et al., 1983). The deduced amino acid sequence was found to encode a polypeptide of 206 amino acids in length with a molecular mass of 22 kDa. This incomplete protein appears to be approximately one third of the determined molecular mass of the fimbrial protein without glycosylation (Castle et al, 1992). The relative abundance of amino acids appears to be similar to those assessed previously (Gardiner, 1985), however, the incomplete nature of the deduced sequence is insufficient for meaningful comparison.

Fimbriae of *U. violacea* have been shown to anchor within the cell as freeze etched preparations of sporidia showed projections of 6 to 7 nm in diameter on the inner surface of the cell membrane (Poon and Day, 1974). The nature of the anchoring of fimbriae to the cell surface has not been investigated. In bacterial pap fimbriae, anchoring and length modulation is made through competitive binding of *Pap* H at the base of fimbriae (Baga et al., 1987). This protein is highly homologous to the structural protein, *Pap* A, except for a cell wall anchoring domain (Baga et al., 1987). It is not clear whether fungal fimbriae require proteins analogous to those of bacteria for attachment to the fungal cell.

The cDNA obtained from *U. violacea* was identified using the polyclonal antiserum raised against the fimbrial proteins of *U. violacea* (Castle, personal communication). This procedure could conceivably identify DNA fragments which encode fimbrial subunits, homologous anchoring proteins or other epitopic proteins. Amino acid sequence comparisons made using the FASTA program yielded a 33 amino acid

sequence which shows similarity to the amino acid sequence of rat leukocyte antigen CD53 (Horejsi and Vlcek, 1991). The area of similarity is a hydrophobic membrane spanning domain (Horejsi and Vlcek, 1991). Thus, this domain may be functionally similar and may be involved in the anchoring of fimbriae to the cell surface. This would be an expected characteristic of either a specific anchoring protein or the fimbrial subunit which itself contains domains required for attachment. For confirmation of pfim3-1 encoding the fimbrial structural protein, the amino acid sequence should be obtained independently from the fimbrial protein in order to compare the sequence to the deduced amino acid sequence.

The strategy employed in the isolation of fimbrial gene sequences from *C. cinereus* was unsuccessful due to incomplete characterization of the fimbrial cDNA of *U. violacea* (pfim 3-1). The sequence showing homology to 16S-like rRNA had not been shown. It is likely possible to use the pfim3-1ES1 fragment to identify sequences showing homology to the fimbrial cDNA without the interference of hybridization to rDNAs. The unpredicted hybridization of pfim3-1 to rDNAs made the isolation of genes from *C. cinereus* unsuccessful, however, in themselves these results are significant. The homology observed between the *U. violacea* fimbrial transcript and 16S-like rDNA may represent a regulatory nucleic acid sequence involved in a mechanism of gene regulation, as yet unidentified.

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